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[GANN, 49, 69~78; June, 1958]

Special Article

CHEMICAL TUMOR PROBLEMS

(Lecture, held in Tokyo, January 24, 1958)

Hans von Euler

Mr. President,

May I express my sincere and devoted thanks to the Japanese Science Council for the kind invitation to give a lecture in this auditory of representatives of so many prominent scientific societies.

In an "initiating phase" of cancerisation normal cells are transformed into cancer cells by a cancerogenic agent. These agents are supposed to attack primarily non-pathogenic selfreproducing substances, for which the term "duplicants" has been proposed (Nothdurft[1]; Butenandt[2]). According to Druckrey[3] a multitude of such duplicants—about 100—are needed for the formation of a cancer cell.

Druckrey and Küpfmüller[4] came in 1948 to the important result that the cancerisation of an organism is an ***additional*** effect, depending on the ***sum*** of all single cancerogenic, irreversible effects, which have occurred in the organism. The same rule is valid for material cancerogenetics and for doses of ionising radiations (Rajewsky).

As regards the chemical reactions between a duplicant and a cancerogenic body very little is known; we may assume that duplicants are macromolecules and thus quite unstable. Many cancerogenes bear more than one active group, a fact, which can cause different sorts of reactions. In many cases the cancerogenic substance is not active before it is transformed in the organism into a working substance. Probably ***several*** reactions participate simultaneously in the cancerisation of a duplicant.

The characteristic disorder and chaotic disorganisation of the metabolism in the cancerous tissue seem to be connected with the cancerous change of the duplicants. ***One*** of these changes consists in the duplicants losing such groups, which determine the rate and the order of the tissue's growth.

Concerning the "promoting phase" of cancerisation, which includes all growth reactions, it is supposed that it can be enhanced by a substance, which itself has no cancerogenic activity, as f. i. croton oil ("conditional cancerogenic substance").

Not all cancer tissues are originated by the same biochemical disturbances. In different tumors different alterations seem to conduct to malignity; most of them are produced by long reaction chains, a fact, which explains the irreversibility of the initiating phase.

The sequence of events, leading to cancerisation can be designed by the following scheme:

Cancerogenic substance+duplicant ⟶ cancerized duplicant

↓

cancer tissue ⟵ cancer cell

A chemical cancerogenic stimulus may be an endogenic ergon (hormone) or an exogenic acting substance.

How the development of the tumor proceeds after the beginning formation of cancerized duplicants (=pathogenic tumorvirus molecules) is not yet sufficiently elucidated. In this development the increase of cancerized duplicants may predominate, at least for a certain period. But in the most cases the increase of the cancer cells seems to continue without the influence of the initiating agent, as it is shown by the "stop experiments".

Now the question arises, whether or not essential differences exist between a cancerized duplicant and such a cell constituent, for which generally the term "tumorvirus" is used.

A virus is characterized by its faculty of selfreproduction, which it obtains by association with a living host-organism—not by combination with an artificial medium. In this symbiosis it is the host which supplies the energy for the growth of the virus; the virus can not produce the growth energy by its own metabolism. In this regard the pathogenicity of the virus is not significant.

Concerning the chemical composition of the animal viruses, which are nucleoproteins, it is well established that the Rous chicken virus contains ribonucleic acid, while the Shope cottontail rabbit virus contains desoxyribonucleic acid. The most complete results on plant viruses (the type of tobacco mosaic virus) have been obtained by Stanley and by Gerh. Schramm. It was not only possible to recognize these viruses as nucleoprotein-derivatives of ribonucleic acid (RNA) but also to elaborate details of their structure. Schramm separated successfully the protein from the RNA *in a serologically active condition: purified it by electrophoresis and* could recombine the components. Schramm's statement that the RNA component recombined with the protein shows the faculty of selfreproduction is of essential interest.

It seems to be an acceptable hypothesis that by the cancerisation of the duplicants or non-pathogenic viruses their factors for differentiation and for growth regulation are distroyed or altered.

Basing on this hypothesis it is our aim, which we follow since many years, to elucidate the chemical reactions, which transform the non-pathogenic duplicants or previruses to tumor-viruses.

By physical, especially electrophoretical, and by chemical experiments carried out on rats bearing chemotumors, Jensen tumors or Yoshida ascites tumors we attempt to establish the differences between normal and cancerous animals relative to their content of free and bound aminoacids, nucleotides, activators, inhibitors and other hormones.

The extraordinary difficulties of this investigation are due to the great number of compounds simultaneously existing in the solutions of homogenates and hydrolysates and to the lack of methods for the separation and analysis of the components. It is not possible to give in the short time of this lecture a detailed description of our analytical procedures. We began our investigation with the determination of some amino acids in the blood of normal and cancerous rats. According to the results obtained by several authors the total content of free amino acids in the blood of cancerous rats is not different from that of normal rats. The increase of the concentration of some of the amino acids in the blood can be accompanied by a simultaneous decrease of some others. The only contribution to this question we were able to find in the literature was given on the concentration of free arginine by Reis, whose method is a modification of Sakaguchi's α-naphtol-method (Biochem. J. Jap. 1925). The results of our histidine determinations carried out on the sera of normal and sarcoma bearing rats are recorded in Table 1.

Table 1. Free Histidine in Serum of Rats

	Age Weeks	Body Weight g	Sarcoma Weight	Days after Implantation	mg (%) Amino-N
Normal	10	120			0.18
	12	123			0.15
	14	170			0.16
	22	265			0.15
	15	150			0.14
Bearing Jensen sarcoma	12	150	31	27	0.09
	12	141	22	27	0.11
	14	140	19	22	0.12
	13	102	5	16	0.09
	12	140	6	16	0.11

As it is seen from Table 1 the average concentration of ***free histidine*** in the serum of Jensen-sarcomatous rats is 33% lower than in the serum of normal rats.

This result is in perfect conformity with the values obtained by Reis for arginine, and they have probably the same cause, namely, the increased requirement of these amino acids in the rapidly growing tumor tissues. L-Histidine-peptide is one of the components of ribonuclease.

Histidine influences the metabolism of animal organs in different ways, in the main by the imidazol group's catalytical action on the hydrolysis of esters. Histidine and tryptophane in this investigation interested us more than other amino acids.

Histidine is decarboxylated to histamine, but we were not able to measure differences of histamine concentration in normal and cancerous blood. Histamine seems to take part in the metabolism as an intermadiate product.

In the present stage of our investigation we consider the hypothesis that histidine or one of its derivatives *is an essential regulator of the metabolism.*

The growth regulating effects of histidine, which are potentiated by ascorbic acid, can be demonstrated by the germination test with barley.

The histidine deficiency revealed in the serum of cancerous rats will perhaps give the key to a complex question raised concerning the metabolism in different stages of cancerisation. But the problem, which inspired a considerable part of our investigation, namely, the reaction between a duplicant or a non-pathogenic virus and a cancerogenic substance, is not yet solved.

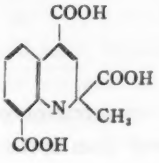
Histidine is one of the substances, which can act, depending on its concentration, as activator or as inhibitor of tumor development, in analogy to phenylurethane, to carcinogenic hydrocarbons (A. Haddow[13]; K. H. Bauer[14]) and to nitrogen mustard and N-oxide mustard (M. Ishidate and T. Yoshida[15]; H. Druckrey[16]).

N-Oxides. Nakahara, Fukuoka and Sugimura[18] made the remarkable observation that 4-nitroquinolin-N-oxide, synthesized by E. Ochiai[17] and his collaborators, has a high cancerogenic activity. It is, as Nakahara[18] emphasizes, the first bicyclic compound, which shows a carcinogenic effect nearly as potent as the most effective polycyclic hydrocarbons.

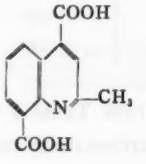
In connection with our studies on quinoline and quinoxaline compounds we[19] prepared quinolines in several different manners:

By 1 molecule anthranilic acid with 2 molecules pyruvic acid 2 quinoline derivatives are formed. One of them, $C_{13}H_{11}O_6N$: Fp 230° (I) is obtained in the form of yellow prisms; the other product (II) is a dicarbonic acid.

I



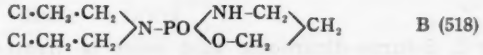
II



Among N-oxides, which we synthesized, I only mention 7, 8-dimethyl-5-nitro-quinoline-N-oxide, $C_{11}H_{10}O_2N_2$, from 3-amino-5-nitro-dimethyl-benzene and glycerol and 5, 6-dimethyl-quinoxaline, of which the two isomers a and b exist[21]

I need not mention, in this connection, the N-oxide mustard bis (β-chloroethyl-) methylamin-oxide (HN 2), which has a large therapeutical value and a low cancerogenic effect, nor the important discovery of Ishidate and Yoshida, who found the singular chemotherapeutic faculty of this substance and its healing effect on different tumors, if they are treated *soon* after the tumor-implantation. It was observed by Ishidate, that this cancer-healing effect is not produced by the administred N-oxide of the methyl-bis-(β-chloroethyl) amine itself; this N-oxide represents—according to Druckrey's[15] and Brock's[16] terminology—only a "transportform" of a still unknown workform.

Another successfully used transportform for nitrogen-mustard has been synthesized by Arnold[22].



B (518)

As regards Bauer's and Haddow's findings mentioned before, that carcinogenic bodies act under certain conditions as tumor inhibitors, it must be noted that this effect does *not* concern the initial phase of cancerisation.

Nucleic acids and nucleoproteins. In connection with our study on chloroplastin, M. Bracco[20], M. L. Stein[22] and myself tried to fractionate nucleic acids and nucleoproteins by precipitation with streptomycin and with other *guanidyl-compounds*[21].

We involved in our studies different *enzymes*, connected with the metabolism of nucleic acids, nucleoproteins, amino acids and their components. We found that desoxyribonucleins from normal rat liver are *dephosphorylated faster by normal rat serum than by carcinomatous serum* (the ratio of hydrolysed PO_4 in 18 hours being 1 : 0.38). I am inclined to consider this result *as an effect of an inhibitor contained in the sarcomatous serum.* Nucleophosphatase preparations are complex enzyme systems; by autodigestion of a nucleophosphatase preparation and elimination of the inhibitor by dialysis it was possible to demonstrate the interaction of a nucleinase with a phospho-mono-estarase in these phosphatase complexes. However we were not able to realize the separation of the nucleinase and the phospho-mono-estarase in nuclei and cytoplasma from normal and cancerous tissues.

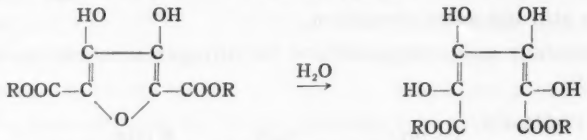
As the cancerisation of normal cells is the consequence of alterations in the normal metabolic reactions and their enzyme systems, we must conclude, *that the enzyme systems, which deal with the mitosis of cancerous cells are different from those dealing with the mitosis of normal cells.*

In this connection the question arose: ***Which chemical reactions determine cell division?***

An essential role in cell division has been attributed to compounds with so-called "bifunctional properties" and especially to substances containing *two functional groups in para-position* as do the hydroquinones.

Under the term "bifunctional substances" Druckrey[23] records compounds of very different structures, such as di-epoxides, disulfonic acid esters and di-amino acids.

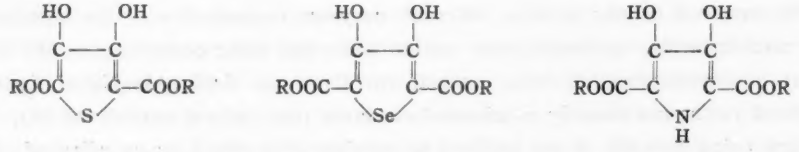
By what kind of chemical reactions the various classes of bifunctional compounds are able to influence cell division seems to be an interesting problem. We therfore synthesized[24] and studied a new type of bifunctional substances.



When a di-hydroxy-2, 5-furan-dicarbonic acid ester is treated with sodium hydrate, the furan-ring is split by hydrolysis and transformed to a chain-molecule, which contains 2 endiol-groups.

This substance was the first diol of this type hitherto known.

By analogous methods we prepared derivatives of thiophene, of selenophene and of pyrole. Also these heterocyclic compounds can be split by hydrolysis to *bis-reductones*. They reduce 2 molecules of the Tillmans reagent, TR, dichlorophenol-indophenol.



Unfortunately the so ubility of our bis-endiols in water until pH 7.5 is very low. It was therefore not possible to carry out complete concentration series with these substances.

With Yoshida ascites-tumor rats the cancer inhibiting effects of these bifunctional substances were measured by two methods:

1) We injected the solution of our substances subcutaneously in the dorsal region a few minutes after the intraperitoneal injection of ascitic fluid, containing a quantity of 100,000–150,000 cells, taken directly from a sarcomatous rat.

2) Or we mixed the fresh ascitic fluid with the solution of our bis-reductones and incubated the mixture at 4° for 20 hours or at 37° for 60–200 minutes.

After daily injections of 1–2 mgs furanester to a rat, we observed a clear but

only weak cancer inhibition. The rats survived 14–35 days, whilst the survival of the control was 5–7 days. We had better results with the thiophene-derivative; After addition of 10 mgs in 5 daily doses, 3 of 11 rats examined survived more than 28 days.

Cancerisation is accompanied by a reduction of the cytochrom system in liver and other organs. I do not wish to give a detailed report of our results, obtained about 15 years ago, concerning the low concentration of the catalysts of the respiration system:

Cytochrome c, cytochrome oxidase, diaphorase and succinic acid in cancerous organs were compared with the concentrations of these ergons in normal organs.

Tissue preparations	Activity per 20 mg prep. Mol 60 H_2/min.		
	Diaphorase	Cytochrome c	Cytochrome system
Heart muscle	4.0×10^{-5}	44.5×10^{-7}	6.5×10^{-5}
Jensen sarcoma	3.8×10^{-6}	2.3×10^{-7}	0

The hepatoma producing effect of butter yellow is essentially retarded by riboflavin. By an interesting dehydrating mechanism riboflavin takes up only 1 H and becomes a free radical, which is stabilized by combination with a protein.

All these results prove that ***great importance should be attached to the supply of free oxygen*** and to ***oxygen-transporters in the preventive and curative therapy of man*** in a precancerous or cancerous condition.

10 years ago Dr. Heller and myself[25] measured the distribution of the catalase activity in the different parts of the normal and of the cancerous liver. In normal rat liver the catalase activity of the cytoplasm is nearly the same in mitochondrias and in the cell fluid containing the microsomes. During the growth of tumors the mitochondrias do not seem to participate in the decrease of catalase activity. First when the rapidly growing tumor has reached a certain volume, the catalase activity of the mitochondria becomes low. By measurements with the Petroff-Hausser counting chamber we found per g rat liver 5.10^{10} mitochondrias.

Catalase and Toxohormone. Low liver catalase activity in cases of cancer was observed in 1910 by Brahn[26] and by Colwell[27]. The fact that the catalaric activity of liver in cancerous rats decreases simultaneously with the components of the cytochrome system is now well established by the experiments of Greenstein[28] and of Nakahara[29] and their schools. This low catalase activity in neoplastic disease is originated by an inhibitor, which is successfully studied by Nakahara and Fukuoka and is called ***toxohormone.***

The isolation and purification methods elaborated by Nakahara yielded very effective toxohormone fractions, as described by Fukuoka and Nakahara[29], by

One, Umeda and Sugimura[30], who also studied the "Kochsaft" factor, present in malignant tumors; they isolated a potent toxohormone preparation from human tumors.

I suppose that the main part of this auditory knows Nakahara and Fukuoka's toxohormone results and I need therefore not refer to the details. According to Nakahara and Fukuoka "there is evidence for assuming that the marked decrease of liver catalase activity in cancer bearing hosts may be a salient expression of a deepseated and more fundamental disturbance, which toxohormone brings about".

The study of known synthetic inhibitors of catalase, as f. i. quinones or the more specific amino-triazoles[31], possibly combined with a "bearer" of protein- or peptide nature, may lead to knowledge of the toxohormone structure.

In 1942 Euler and G. de Hevesy[32] isolated catalase from irradiated and non-irradiated muscle of normal rats. The activity of the enzymes studied (catalase and nuclease) *increased* after irradiation with 3000r. The catalase reaction constant was $k \cdot 10^3 = 25.2$ before irradiation and 38.5 after irradiation, increase of k being 53%.

The nuclease activity, measured by the amount of desoxiribonucleic acid split in 4 hours was 46% before irradiation and 66% after irradiation.

The values obtained in other experiments for the decrease of enzymic activities after irradiation are in many cases the resultants of the activating and the inactivating effects of the Roentgen rays. The mechanisms of the two effects are probably very different.

Experiments carried out in my institute and the more extended investigation by Arne Forssberg on the radio-sensitivity of catalase in vivo support the view that the enzymes are extremely radio-resistant in vivo, in contrast to the sensitivity of purified enzymes in aqueous solution.

In connection with the results obtained with L. Ahlström and G. de Hevesy[33] about the influence of Roentgen rays on the nucleic acid metabolism in cancerous and in normal tissues it was of interest to establish the influence of Roentgen rays on the toxohormone. We repeated therefore our earlier experiments, carried out with Jensen-sarcoma and with liver tissues from normal and from Jensen-sarcoma bearing rats: There was no indication for a radio-sensitivity of a toxohormone, contained in an extract, which was prepared and tested according to the method described by Nakahara and Fukuoka[34]

Several authors had stated in 1941 *a serological* organ specifity of the protoplasma particles, which were prepared from organ homogenates by high speed centrifuging. These particles contained both *unspecific* antigens, detectable in many other organs, and also antigens, *specific* for the studied organ.

In an investigation, carried out by Weiler[35] in Butenandt's institute, it was stated, some years ago, that, with progressive cancerisation, the microsome fraction of rat liver cells loses its organ-specific antigen-qualities. His experiments seem to show that the boundless division of cancer cells is only a secondary consequence of a defect, which follows the cancerisation. Together with Friedrich-Freksa, Weiler developed antibodies against the organ-specific antigens of the normal rat liver and coupled them with fluorescein. The dyestuff-marked antibodies were bound *only by the normal liver cells*, but *not* by the *cancer cells* of the liver.

It is possible that in the present period of cancer research essential progresses can be reached by immunological and serological experiments.

By this consideration my thoughts are directed to an earlier period. About thirty years ago, I was also kept by serological conjectures[36]. It would be of little interest for you to hear of them. But in science as in the whole life it happens that one's plans and aims are realized in the next generation. At that time—it was in 1927—working in my University's rather insufficient chemical institute I had the priviledge to receive a prominent guest from Japan: Dr. Kojima. A short time later by the support of the Wallenberg and the Rockefeller Foundations I was enabled to continue my research work on a larger scale, to my sorrow without Dr. Kojima, who then had returned to Japan.

I shall not recollect the plans and enthusiastic discussions I had with my colleague Kojima. I shall only take the opportunity I have to-day to express my sincere and cordial thanks for a year of collaboration and personal intimate relations, and express my hope that such relations between the Japanese and the Swedish institutes and schools, initiated by Dr. Kojima's visit to Stockholm, may last and intensify.

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[GANN, 49, 79~84; June, 1958]

STUDY ON THE NATURE OF BINDING OF DIMETHYL-AMINOAZOBENZENE WITH LIVER PROTEINS IN RATS FED THE CARCINOGEN

II. SYNTHESIS OF THE AMINO ACID BOUND AMINOAZO DYE

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INTRODUCTION

In the precancerous stage, the chemical binding of the carcinogenic aminoazo dye with liver proteins of the experimental animals has been thought a cause of the transformation of normal cells into cancerous cells (1). Since the amount of the polar dye available from rats liver fed DAB* was too small to be studied satisfactorily, the synthesis of polar dye-like substances having same assumed chemical structures and the comparison of properties between the natural and synthetic polar dyes were considered necessary for elucidating the chemical nature of the binding in the polar dye obtained from the dye-bound liver proteins. In this paper the Mannich reaction (2) (3) between the aminoazo dyes and several amino acids will be discussed.

EXPERIMENTAL

I. Preliminary Experiment.

According to the above idea, the Mannich reaction between several amino acids and AB or MAB has been investigated.

Among the amino acids investigated, only tyrosine, histidine, and tryptophan gave polar dyes under the experimental condition described below. DAB did not give any appreciable amount of the polar dye. Phenylalanine and cysteine as well as other amino acids failed to produce polar dyes. It is assumed therefore that addition of the aromatic amino acids at the benzene ring of the aminoazo dye is not the main reaction concerned, but substitution at the amino group is predominant. An example of the reaction condition is shown here:

* The following abbreviations were used in this paper:
AB: aminoazobenzen, MAB: N-methylaminoazobenzene, DAB: dimethylamino-azobenzene

AB (or MAB) alcohol solution (5 to 6mg, 6×10^{-3}M) 5cc
Amino acid in 2% $NaHCO_3$ solution (12×10^{-3}M) 5cc
$NaHCO_3$ solution (2%) 2cc
Formalin solution (35%) 1cc

The above mixture was incubated at 35°C over night. The mixture was extracted several times with benzene to remove the non-polar dye. The faintly yellowish aqueous solution was acidified with HCl and submitted to the spectrophotometric colorimetry at 520mμ using DAB as the standard.

Table 1

Reaction	Components	Yield of Polar Dye (expressed as DAB)
Dye	Amino Acid	
AB	Try	82
AB	His	20
AB	Try	Trace, unestimated
MAB	Tyr	53
MAB	His	17
MAB	Try	Trace, unestimated

(unit : μg)

The yields of the polar dyes thus prepared are shown in the following table, although they are a mixture of the several Mannich products. (Table 1)

II. Preparation of Crystalline AB-Tyrosine Polar Dye.

The polar dye prepared from tyrosine, aminoazobenzene and formalin has been shown to be a mixture of various reaction products chromatographically as well as spectrally. There are many possibilities of the reaction available for these reaction components. The mixture of the reaction products could be further fractionated chromatographically. A fraction which behaves in the similar ways chromatographically to the natural polar dye was separated in the following way. After extracting the non-polar dye from the reaction mixture with benzene, the polar dyes were extracted with n-butanol. The butanol extract was washed with water and then evaporated under reduced pressure. The residue was dissolved in N-HCl and the pink solution was submitted to IRC-50 (H-form) chromatography. The absorbed dye was washed with N-HCl, water and 30% alcohol and finally eluted with 60% alcohol. The 60% alcohol eluate was then submitted to the Brockmann alumina column chromatography. The alumina which had been treated previously with diluted HCl was used. The dye was eluted with water. The crystallization of the AB-Tyr polar dye thus prepared was unsuccessful.

In order to avoid the complexity in reactions due to free tyrosine, the aminoazo dye was first introduced to tyrosine residues of a protein through the Mannich reaction. The dye bound protein was then subjected to hydrolysis. Out of the hydrolysate the amino acid bound dye was extracted and purified as described above. At first a crystalline serum albumin (bovine) was used for the preparation, but the bovine serum albumin contains histidine, tryptophan as well as tyrosine, which are able to combine with aminoazobenzene dyes through the Mannich reaction. During the course of the preparation of the polar dye, the major part

of the polar dyes containing histidine and tryptophan seemed to be destroyed because they were labile compared to the polar dye containing tyrosine. This complexity could be eliminated by using silk fibroin which is rich in tyrosine content but is devoid of histidine and tryptophan.

Silk was first washed with hot water several times to remove sericin and dried. The sericin free fibroin (2g) was immersed in 16ml of 50% sulfuric acid at room temperature and kneaded untill the mass became pasty. Immediately after the fibroin was dissolved into a swollen pasty mass, the whole solution was mixed with ten times as much of alcohol. The fine fibroin gel thus precipitated was washed with alcohol untill the acidity of the washing alcohol became weak. The amorphous fibroin thus prepared was finely suspended in 200ml of 0.1M phosphate buffer (pH 7) and the pH was adjusted. 0.1g of AB (or MAB) was dissolved in a small amount of alcohol and poured into the fibroin suspension under stirring. Finally 2ml of the neutralized formalin (35%) was added. The mixture was incubated at 35°C for a few days. The AB bound fibroin was precipitated by centrifugation, washed with 50% alcohol, alcohol and ether successively. The dried AB bound fibroin powder was then subjected to a hydrolysis. The alcoholic alkaline hydrolysis for 20 hours according to Millers (1) was tried, because the fibroin was not attacked by the *Slreptomyces griseus* protease (4). The polar dye was extracted and purified as described above. The water eluate from the Brockmann alumina (pH 3.6) was kept at room temperature and fine needle crystalls appeared after several hours. The crystals were separated and washed quickly with distilled water.

The crystalline polar dye thus obtained is slightly soluble in distilled water but hardly soluble in slightly acidic water (pH 3.6). It is easily soluble in strong acidic as well as in alkaline water. It is insoluble in benzene and petroleum ether as well as other nonpolar solvents. It is also hardly soluble in alcohol, but upon adding acid or alkali it dissolves in aqueous alcohol. The picture of the crystals is shown in Fig. 1.

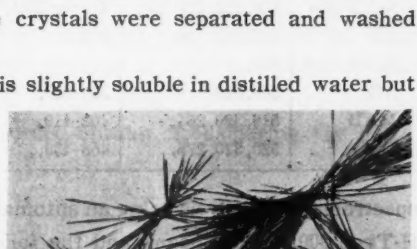

Fig. 1. Picture yf Crystalline AB-Tyr polar dye.

III. Other Synthetic Polar Dyes.

MAB-Tyr polar dye was prepared and fractionated similarly as described for AB-Tyr polar dye. This compound was rather unstable compared with AB-Tyr polar dye. It was decomposed to MAB and tyrosine derivative after several days of standing at room temperature. MAB-Tyr polar dye has not been crystallised so far.

AB-His, AB-Try, MAB-His and MAB-Try were also prepared. These non-tyrosine polar dyes were, however, very unstable and could not be subjected to the chromatographic separations. The absorption spectra were measured immediately after the polar dyes were extracted with butanol.

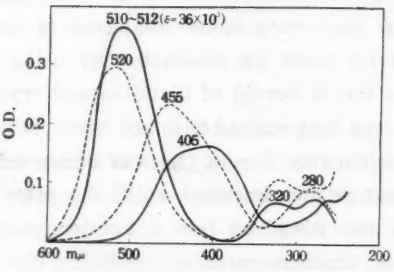

Fig. 2 Spectra of Synthetic AB-Tyr and MAB-Tyr Polar Dyes. Solid curves ; AB-Tyr polar dye
Dotted curves ; MAB-Tyr polar dye

IV. Spectra of Synthetic Polar Dyes.

Absorption spectra of several synthetic polar dyes were measured in alcoholic acid or alkaline media. Spectral characteristics of the synthetic polar dyes are shown in Table 2. The spectral curves of AB-Tyr and MAB-Tyr are illustrated in Fig. 2. The absorption spectra of AB-Tyr polar dyes resemble with that of MAB, so does that of MAB-Tyr with DAB. This may also suggest that the tyrosine-binding takes place at the amino group of the dye.

Table 2

Dye	Absorption Maximum (mμ)	
	Acidic	Alkaline
AB-Tyr	510, 320, 270,	430, 270
MAB-Tyr	520, 320, 280,	455, 280
AB-His	505, 320,	
MAB-His	510, 320, 270-290,	410 (or 398), 270
AB-Try	507, 370, 320	385
MAB-Try	514,	
AB	500, 320	380-383, 250
MAB	510, 320, 264,	402-410, 255
DAB	520, 320, 275,	408-430, 260

V. Chemical Structure of Synthetic AB-Tyr Polar Dye.

The crystalline AB-Tyr polar dye was subjected to the infra-red spectrometric analysis using an automatic recording infra-red spectrometer, (Hilger H-Type 800, double beam) and the following characteristic absorptions were confirmed among many other absorptions.

$-N^+H_3$ (3250cm^{-1}), -OH (3417cm^{-1}), and $-COO^-$ (1627 and 1327cm^{-1})

The elementary analysis (C and H) was also carried out in our laboratory and the values of C: 67.74%, H: 5.88% and N: 13.95% were obtained. Those were compared with the following calculated values. (Table 3)

The above analytical data correspond quite well to the unimolecular combination between aminoazo dye and tyrosine. This assumption was also confirmed by the reaction with nitrous acid. Upon the additon of a very small amount of sodium nitrite to the acidic solution of the polar dye, the pink color of the dye disappeared instantly. This means that the amino group at position 4 is not a tertially amino

group which does not react with nitrous acid. After the addition of ammonium sulfate, the solution was heated in a water bath for a few minutes. Development of the slight pink color was observed. The reappearing of the pink color upon heating is due to the decomposition of the nitroso compound which was formed by the reaction of the secondary amino group of the dye with nitrous acid. This reaction may be considered as a proof that the amino group is a secondary one.

Table 3

Mol. ratio (AB : Tyr)	Mol. formula	Composition calcd.		
		C%	H%	N%
1 : 1	$C_{22}H_{21}N_4O_3$	67.69	5.64	14.35
1 : 2	$C_{22}H_{22}N_5O_6$	65.87	5.65	12.01
2 : 1	$C_{35}H_{33}N_7O_2$	70.12	5.51	16.36

A quantitative assay was also tried, using the Folin-Ciocalteau phenol reagent (5) (6). The optical density of the blue color developed by the reagent was compared at 600mμ using tyrosine as the standard. The determination of the aminoazo dye moiety was carried out by comparing the optical density at 520mμ in the acidic solution using DAB as the standard. The molar ratio of dye to tyrosine residue thus obtained was 0.5. This, however, does not necessarily mean that one molecule of dye is bound with two molecules of tyrosine, because the intensity of the blue color due to the Folin reaction product may be greatly enhanced by a substitution at one of the ortho positions of the tyrosine residue, as shown by Fraenkel-Conrat et al. (3). With AB-Tyr polar dyes derived from AB and free tyrosine, the two values were obtained, one 0.5 and the other 1.0. If we multiply the observed dye/Tyr ratio by factor 2, assuming that the absorption of Folin reaction product of the one ortho substituted tyrosine is twice as strong as that of unsubstituted tyrosine, we can get the unimolecular stoichiometry for the crystalline AB-Tyr polar dye derived from silk fibroin. Considering the steric difficulty, the possibility for two residues of tyrosine in the fibroin to combine with one molecule of AB is very little. Multiplying the molar ratio observed optically using tyrosine as the standard by factor 2, therefore, may be justified from the above several reasons. The above analytical data, spectral data as well as the consideration of the Mannich reaction mechanism may suggest that the structure of the synthetic AB-Tyr polar dye may be as described in Fig. 3.

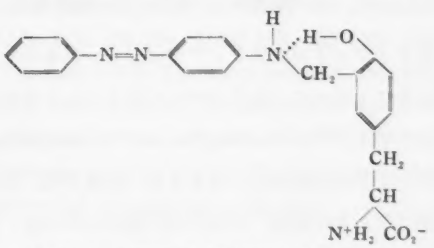

Fig. 3 Assumed Chemical Structure of Crystalline AB-Tyr Polar Dye

SUMMARY

Crystalline aminoazobenzene tyrosine compound was prepared by hydrolyzing

aminoazobenzene dye bound silk fibroin, which was prepared through the Mannich reaction. The chemical structure of the synthetic polar dye thus obtained has been investigated from the UV and IR absorption spectra, elementary analysis, reaction with nitrous acid as well as with Folin-Ciocalteau's reagent. The unimolecular combination between the 4-position amino group of aminoazobenzene and one of the ortho position of tyrosine through a methylene bridge has been assumed.

ACKNOWLEDGEMENT

The authors are grateful to Prof. S. Akabori, Dr. W. Nakahara, and Mr. A. Hanaki for their encouragement and helpful advice throughout the course of the study. The infra-red spectrum was taken by Mr. G. Chihara, and the elementary analysis was carried out by Mr. S. Masuda, to whom the authors' thanks are also due. A part of the expenditure for the study was paid from the Scientific Research Fund from the Ministry of Education, Japan.

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要　　旨

アミノアゾベンゼンの肝臓蛋白との結合に関する研究
第二報　アゾ色素-アミノ酸結合体の合成

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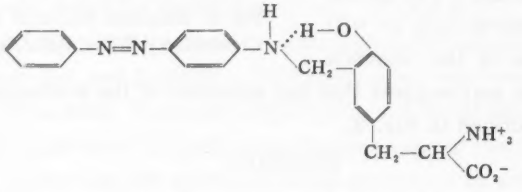
絹フィブロインにアミノアゾベンゼンをホルマリンを用いて結合させ，生じたアミノアゾ色素結合フィブロインをアルコール性アルカリで加水分解した後，抽出，柱クロマトグラフ法等によりアミノアゾベンゼンチロシンの化合物を結晶状に調製した。この化合物の性質を，可視，紫外および赤外線吸収スペクトル，元素分析，亜硝酸との反応，フォリンのフェノール試薬等で調べた。その結果この化合物の構造は次の如く決定した。　(文部省科学研究費による)



[GANN, 49, 85~95; June, 1958]

STUDY ON THE NATURE OF BINDING OF DIMETHYL-AMINOAZOBENE WITH LIVER PROTEINS IN RATS FED THE CARCINOGEN

III. COMPARISON OF THE PROPERTIES OF NATURAL AND SYNTHETIC POLAR DYES

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INTRODUCTION

Since the first paper (1) of ours on the isolation and purification of the amino acid bound aminoazobenzene dye (natural polar dye) from rats liver fed DAB was sent for the publication, a few improvements have been added to the procedure. The natural polar dye reported in the preceding paper was still contaminated with strong fluorescent substances. These contaminations as well as the optical densities in the UV absorption spectra were greatly reduced with our new samples prepared by the improved method. The careful column chromatography using IRC-50 (H form) has revealed that there are at least two components in the natural polar dye.

In the second paper preceding this (2) we have described the synthesis of aminoazobenzene bound amino acids. The synthetic polar dyes prepared by the Mannich reaction were the following: AB-Tyr, MAB-Tyr, AB-His, MAB-His, AB-Try, and MAB-Try. Out of the above, only AB-Tyr and MAB-Tyr were rather stable and somewhat resembled the natural polar dyes, especially in the absorption spectra, electophoretic mobility, etc. Careful comparison of the properties of the natural polar dyes with the synthetic polar dyes, however, has revealed very interesting informations concerning the chemical structure of the natural polar dye.

EXPERIMENTAL

I. Improved Method for Preparation and Purification of the Polar Dye from Liver of Rats fed DAB.

The liver of rats fed DAB for one month was homogenised in Waring blendor in the presence of equal volume of water soon after the animals were sacrificed by decapitation. Equal volume of methanol was added to the homogenate and the

mixture was heated in a boiling water bath under stirring for several minutes. The coagulate was precipitated by centrifugation, washed a few times with methanol and twice with acetone, and finally with ether. The defatted liver powder thus prepared was subjected to the enzymatic hydrolysis using a strong protease from **Streptomyces griseus** (3). The fine liver powder was suspended in ten to twenty times as much of 0.1 M sodium acetate solution and pH was adjusted to 7 using sodium carbonate (2%) if necessary. A small amount of the protease (about one fiftieth of the weight of the liver powder) was added. It was incubated in the presence of toluene at 35°C for several days. During that period pH was checked once in a while and readjusted to 7. By the end of the incubation period (about 5 days) most of the solid material disappeared into a brownish solution. After heated in a boiling water bath, the solution was filtered through cotton. The brownish clear filtrate was extracted twice with benzene (or petroleum ether) to remove a trace of non-polar dye, the remaining polar dye was extracted with n-butanol from the aqueous solution. The yellow butanol extracts were combined and evaporated under the reduced pressure. The residue was dissolved in 1N hydrochloric acid and ethyl acetate. The polar dyes were extracted from the yellow ethyl acetate solution by shaking with 1N hydrochloric acid solution. The pink hydrochloric acid solutions containing polar dyes were combined. After the dissolved ethyl acetate was driven off under reduced pressure, the solution was submitted to the first chromatography using IRC-50 (H form, 100-200 mesh). The polar dye was adsorbed at the top of the column. After washing with N hydrochloric acid, water, and 30 per cent alcohol in succession, the polar dye could be eluted with 60 per cent alcohol. Using a fraction collector, the 60 per cent eluate was fractionated into three main portions; i. e. Fr. 1: swiftly moving, Fr. 2: slowly moving and Fr. 3: the last slowly moving fraction. After Fr. 3 was collected, the column was eluted with 2N ammonia, and Fr. 4 was obtained. This Fr. 4 could be again subjected to the hydrolysis and purification.

The absorption spectra of Fr. 1, 2 and 3 were measured. Fr. 1 appeared to be contaminated with non-polar dye but the amount was very small. Fr. 2 and 3 seemed to be the main components and rather pure. Fr. 2 showed maximum absorption at 522mμ and Fr. 3 at 514mμ. The amount of Fr. 2 was small compared with Fr. 3 and therefor might have been overlooked in the first paper (1). These 60 per cent alcohol eluates (Fr. 2 and 3) were chromatographed again using hydrochloric acid pretreated Brockmann alumina (4). After the column was washed with 60 per cent alcohol, the adsorbed dye was eluted with water. The aqueous eluate was mixed with equal volume of 2N sulfuric acid and refluxed in a water bath for a few hours (caution: not too long). The pink sulfuric acid solution was then rechromatographed with IRC-50 (H form) and the 60 per cent alcohol eluate was

collected in the same way as described above. 60 per cent eluate was then subjected to the alumina chromatography again and the water eluate was collected. The treatment with 1N sulfuric acid, followed by IRC-50 and alumina rechromatographies has been proved effective for eliminating contamination, probably due to breaking some labile bonds of the contaminants. The products thus prepared will be called "Fr. 2 (or 3), sulfuric acid treated" in this paper, and are probably of the highest purity up to the present.

II. Spectra of Polar Dyes

Spectra of the sulfuric acid treated and untreated natural polar dyes (Fr. 2) are shown in Fig. 1. The spectral character of both synthetic and natural polar dyes are listed in Table 3.

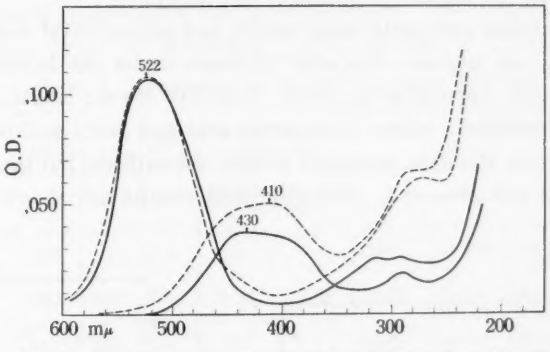


Fig. 1 Absorption Spectra of Natural Polar Dye (Fr. 2)
Solid curves; N-H_2SO_4 treated
Dotted curves; N-H_2SO_4 untreated

III. The Presence of Phenolic Hydroxyl Group in Polar Dye

The Folin-Ciocalteau phenol reagent (5) which is specific to the phenolic hydroxyl group and develops the intense blue color was used for measuring the presence of phenolic hydroxyl group in the synthetic and natural polar dyes quantitatively as well as qualitatively. The general analytical procedure has been described by R. M. Herriott (6). The substances listed in Table 1 were investigated.

Table 1. The Presence of Phenolic Hydroxyl Group in Polar Dyes

Substance	Folin-Ciocalteau's reaction
Natural polar dye (enzymatically prepared)	Positive
Natural polar dye (alkali treated)	Positive
Natural polar dye (Fr. 2, sulfuric acid treated)	Positive
Synthetic AB-Tyr polar dye (crystalline)	Positive
Synthetic AB-His polar dye	Negative
Synthetic AB-Try polar dye	Negative

For the quantitative purpose of measuring tyrosine residue, the crystalline synthetic AB-Tyr polar dye (2) was used as the standard, the intensity of the absorption at 600 mμ was compared with the aid of the Beckmann spectrophotometer. The molar ratio of phenolic hydroxyl (tyrosine) to aminoazo dye moiety was calculated. The amount of dye moiety was obtained by comparing the absorption with DAB at 520 mμ. The ratio Dye/Tyr was 1.0 for Fr. 2 (sulfuric acid treated). The above results may suggest that one mole of dye is bound to one mole of tyrosine residue probably at the ortho-position of the tyrosine residue.

IV. Paper Electropheretic Behavior of Polar Dyes

The paper electrophoretic behavior of natural and synthetic polar dyes were studied. In this experiment, unfractionated synthetic polar dyes were used since it was carried out at the earlier stage of the study.

At pH 6.8 (lutidine and acetic acid buffer) and pH 4.2 (0.1M acetate buffer) the polar dyes did not migrate even after 10 hours under the following conditions: 500 volts, 10-20mA, Toyo filter paper No 51 (width 10 cm ; length 22 cm).

At lower pH condition, where 0.1M acetic acid and 0.01N hydrochloric acid was used as the solvent the dyes migrated toward the cathode, but the severe destruction of the dyes was observed. The migration results are shown in Table 2.

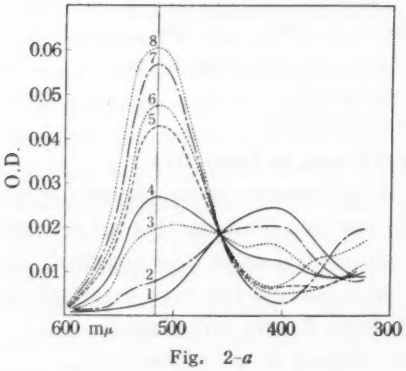


Fig. 2-*a*

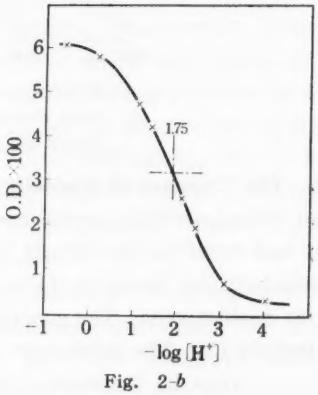


Fig. 2-*b*

Fig. 2-a pH-Dependency of Absorption Curves of Natural Polar Dye (Fr. 2)

Curve No.	pH of the medium	Curve No.	pH of the medium
1	3.97	5	1.29
2	3.03	6	0.96
3	2.33	7	0.0
4	2.02	8	−0.8

Fig. 2-b pH-Dependency of Intensities of Absorption of Natural Polar Dyes (Fr. 2) at 516mμ

Table 2. Paper Electrophoretic Behavior of Polar Dyes

Substances	Electrophoretic Migration (cm)	
	0.1N Acetic acid (2 hours)	0.01N Hydrochloric acid (10 minutes)
Natural polar dye (unfractd.)	3.1	1.8
AB-Tyr (synthetic)	3.3	1.8
MAB-Tyr (synthetic)	3.6	2.0
MAB-His (synthetic)	5.0	3.5

V. Paper Chromatography of Polar Dyes

The paper chromatography was tried for the polar dyes as well as for nonpolar dyes, using Toyo filter paper No. 51. The dyes were developed with the ascending technique at room temperature for about 16 hours. Two solvents systems were used; the upper layer of n-butanol: 0.75N ammonia (1 : 1) and the mixture of

Table 3. Comparison of Several Properties of Dyes

Dyes	Max. of Absorp.(mμ)		pK'a	Rf of Paper Chromato.		Color change with HNO_2*			Stability against H_2SO_4
	Q	K		BuOH: NH_4OH	AcOH: EtOH	I	II	III	
Natural P.D. (alkali, unfr.)	516	—	2.1	—	—	pink	yellow	light red	stable
Natural P.D. (Fr. 2)	522	430	1.6-1.75	0.63	0.36	pink	light yellow	light red	stable
Natural P.D. (Fr. 3)	514	410	—	—	—	pink	light yellow	light yellow	stable
AB-Tyr P.D.	510	400	2.6	0.50	0.33	pink	light yellow	decomp.	decomp.
MAB-Tyr P.D.	520	440-450	3.0	0.63	0.53	pink	pink	decomp.	decomp.
AB	500	380-383	3.65	1.00	0.50	orange	yellow	yellow	stable
MAB	508	402-410	3.1	1.00	0.46	pink	light yellow	pink	stable
DAB	520	408-430	3.65	1.00	0.36	pink	pink	pink	stable
3-Me-AB	508**					orange	light yellow	light yellow	stable
3-Me-MAB	514**					red	light yellow	red	stable
3-Me-DAB	none	360				color-less	color-less	color-less	stable
2-Me-AB	502**								
2-Me-MAB	508**								
2-Me-DAB	520**								

* I; Before the addition of HNO_2 II; Upon the addition of HNO_2 III; Upon heating

** Cited from Miller et al. data (11)

2/3M acetic acid : ethanol (2 : 1). The values of Rf observed are shown in Table 3.

VI. Solubility of Polar Dyes

Natural and synthetic polar dyes are soluble in water and alcohol but insoluble in benzene and petroleum ether. AB, MAB and DAB, however, shows the different solubility behavior.

VII. pH-Color Relation of the Non-polar Dyes

Absorption spectra of the natural and the synthetic polar dyes were measured at different values of pH in the presence of hydrochloric acid. The maximum absorption of Q band was plotted against pH. The value of pH at the inflexion point on pH-optical density (at the maximum of Q band) curves corresponds to pK'a of the azonium form of the dyes. pH was measured with the aid of the glass electrode pH meter.

Fig. 2 showed the spectral change of the natural polar dye (Fr. 2) according to the pH values of the medium. The values of the pK'a of the dyes thus obtained are compared in Table 3.

VIII. Reaction with Nitrous Acid

In order to examine the nature of the amino group at the position 4 of the dye a small amount of nitrous acid was added to the acidic solution of dyes and the color change of the dyes was observed. If primary amino group is present at the position 4, for instances, as in AB, 3-Me-AB, etc., it is changed to hydroxyl group through diazonium form and the color of the solution changes almost immediately from red to light yellow. The heating of the solution does not make the red tone reappear.

If the dye has the secondary amino group (MAB, 3-Me-MAB, etc.), it becomes the nitroso compound and the pink color disappears quickly. But when the solution is heated in the water bath for a few minutes, the nitroso compound is decomposed and a part of it returns back to the original secondary amino compound, thus reappearing the slight pink color.

If the dye has the tertiary amino group, the addition of a small amount of nitrous acid does not change the color of the solution because the tertiary amino group does not react with nitrous acid. DAB belongs to this group.

The observation obtained with non-polar dyes was applied to the synthetic and the natural polar dyes to see the nature of the amino group in the dye moiety. The results are summarized in Table 3. It turned out to be that the crystalline synthetic AB-Tyr polar dye has secondary amino group although in this case the reappearing of the pink color upon heating was weak, probably due to the cleavage of the substitution at the amino group. The addition of ammonium compounds before heating the solution may improve the reappearing of the color. The natural polar dyes seem to be a mixture of the primary and the secondary amino dyes.

The Fr. 2, which is eluated faster than the Fr. 3 in IRC-50 column chromatography and has the maximum absorption at 522 mμ showed the secondary amino character, while the Fr. 3 which has a maximum absorption at 514 mμ showed the primary amino nature.

The pink color of the unfractionated natural polar dye disappeared completely upon the addition of nitrous acid, and this suggests that there is no possibility that the natural polar dyes contain dimethyl amino group derived from DAB.

IX. Stability against the Treatment with 1N Sulfuric Acid

When the synthetic AB-Tyr or MAB-Tyr polar dyes were refluxed in the presence of 1N sulfuric acid for a few hours, AB or MAB was recovered almost quantitatively. The tyrosine was not identified in the hydrolysate, but a certain ninhydrin positive substance was detected and it was assumed to be a derivative of tyrosine. When the same treatment was applied to the natural polar dye, no non-polar dye was produced even after 5 or 6 hours of heating. This may suggest that the type of the substitution is different between the synthetic and the natural polar dyes.

X. Methylation of Amino Group of Polar Dyes

Out of the results obtained above it seems likely that the substitution is not at the amino group of the dye moiety but at some other place in the benzene ring of the natural polar dyes. There are two main possibilities concerning the point of the substitution, i. e. position 2 and position 3. Miller and their group recently (7, 8, 9) switched to the idea that the binding may occur at position 2 through an unidentified group. They had been assuming the Mannich type of binding before (1). Nye et al. (10) also considered the substitution at the position 2 probably through amino group mainly from the spectral analysis.

From a view point of organic chemistry, the reactivity of position 2 seems to be much weaker compared with that of position 3. Since we do not have enough amount of the purified polar dye to test this problem we could not try the reductive cleavage of the natural polar dye and analyse the products. However, the metylation of the polar dyes and the spectral change hereafter appear to give a clue to the answer of the problem. It is known that 3-Me-DAB does not show appreciable pink color in acidic solution due to the disappearnce of Q band, which is explained by the steric interferance between the dimethylamino group and the position 3 substituent, but 2-Me-DAB shows intense pink color (11, 12).

Out of the various methylating reagests, the formalin and formic acid mixture was shown to be ideal because it reacts only with amino group and the methylation stops at the stage of the tertiary amino group.

Before applying this reaction to the natural polar dye, 3-Me-MAB was tried. 3-Me-MAB was refluxed with 5ml of formic acid and 2.5ml of formalin for 5 to 6 hours. After the reaction is over, the mixture was diluted with water and the dye

was extracted with petroleum ether. Neutralisation of the solution may fascilitate the extraction of the dye by petroleum ether. The petroleum ether extract was washed several times with water and then subjected to the Brockmann alumina column chromatography. Two bands were separated by eluting with petroleum ether. The main band which moves faster has been shown the methylated product, 3-Me-DAB, and the minor, slowly moving band the unreacted 3-Me-MAB. The spectral measurement and nitrous acid tests described above were carried out for these fractions (see Table 3). 3-Me-DAB thus obtained shows a strong absorption at 320mμ in acidic solution, but no Q band (the vicinity of 520mμ). In alkaline medium, absorption maximum at 360mμ and 260mμ were observed. The natural polar dye was methylated similarly using one ml of formic acid and 0.5 ml of formalin. The reaction mixture was refluxed for three different periods, two, four and six hours. During the time the color changed from pink to slight yellow. After the reaction is through the mixture is diluted with water and extracted with benzene to remove a trace of nonpolar dye and then extracted with n-butanol. The butanol was washed with water, ammonia and water again. Butanol was evaporated under the reduced pressure, and the residue was dissolved in a small volume of alcohol. The color of the alcoholic solution was yellow. Upon acidifying the alcoholic solution with hydrochloric acid, slight pink color was observed only for the product of two hours reaction, but no pink color developed for both four and six hours reaction products. Moreover, the yellowish color was diminished by the addition of acid.

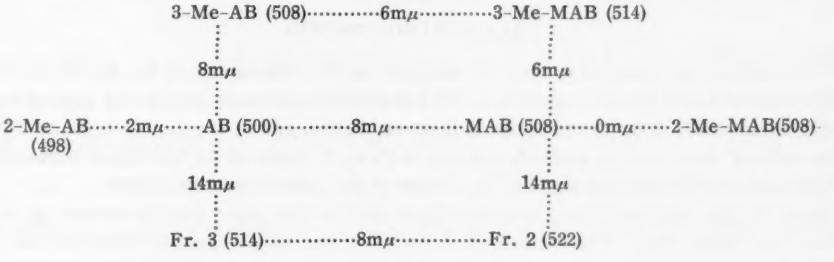
Discussion

An amino acid which is responsible for binding the aminoazo dye in the natural polar dye may be tyrosine. The purified natural polar dye shows intense blue color by the Folin-Ciocalteau phenol reagent. The stoichiometry between the dye and the tyrosine was shown to be unimolecular. The ninhydrin reaction of the polar dye was not sensitive from some unknown reasons, but it becomes positive after it was hydrolysed with 6N hydrochloric acid for 20 hours. We could not identify any known amino acid out of the hydrolysate though. This ninhydrin positive substance might be a derivative of tyrosine.

The possibility that the tyrosine derivative is bound with the dye at the position 4 amino group is very slight. The substitution of the big molecule at position 4 amino group seems to be easily decomposed by heating with acid as shown with the synthetic AB-Tyr polar dye. As to a problem whether the substitution takes place at the position 2 or the position 3, we can not answer it definitely, but we are rather inclined to assume that the substitution may take place at the position 3 from the reason described above.

As to the nature of position 4 amino group we assume that it is either primary or secondary amino group. We could not find any evidence to support the presence of dimethyl amino group using our samples. If our finding is right, the N-methyl group is partially or completely demethylated before the dye combines with the tyrosine residue of a special protein in the rat liver. Otherwise there may be another possibility that the Mannich type products which have been discussed in the preceding paper are the first binding product, and the tyrosine residue is later transfered to the position 3, producing the stable polar dyes described in this paper. If our imagination is permitted to go further, in the case of the first hypothesis the decomposed N-methyl radicals are once converted to formalin (oxidative demethylation) and may contribute to the binding of the two components (dye and tyrosine) through a rather stable C-C-C binding in the polar dye (Fig. 3).

Table 4. Relation among the Maximum Absorptions of Structually Related Dyes



It is very interesting to note that there is a regular displacement in the absorption maximum of Q hand between AB, MAB; 3-Me-AB, 3-Me-MAB; 2-Me-AB, 2-Me-MAB; natural polar dye (Fr. 3) and natural polar dye (Fr. 2) as shown in Table 4.

From the results described above, we may present tentative chemical structures of the natural polar dyes as shown in Fig. 3.

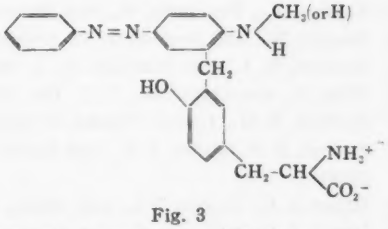


Fig. 3

Fig. 3 Assumed Chemical Structure for the Natural Polar Dyes

There is a possibility that the methylene bridge between the two benzene rings might have been converted to carbonyl, because it is known to be easily oxidized. The displacement of the Q band toward the longer wave-length may be rationalised by assuming the presence of carbonyl between the rings thus permitting the hyperconjugation of the double bonds.

Summary

1. The method of isolation and purification of the amino acid bound aminoazobenzene dye (polar dye) from rats liver fed DAB was improved and highly purified preparations having different absorption maxima have been obtained : 514 and 522mμ.

2. Several properties such as optical absorption spectra, Rf, electrophoretic mobility, stability against acid, reaction with nitrous acid, methylation, pH-color dependence, etc. of the natural polar dyes were investigated in comparison with those of non-polar aminoazo dyes as well as the synthetic polar dyes having amino acid substitution at the position 4 amino group.

3. The chemical structure of the natural polar dyes have been assumed tentatively as follows: a substituent containing tyrosine residue is located at the position 3 of aminoazo dye moiety, and the amino group at the position 4 is either primary or secondary, but not tertiary.

Acknowledgements

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要　旨

アミノアゾベンゼンの肝臓蛋白との結合に関する研究
第三報　天然アミノアゾ色素－アミノ酸結合体と合成アミノアゾ色素－チロシン結合体との性質の比較

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ヂメチルアミノアゾベンゼン投与の鼠肝臓蛋白よりアミノアゾ色素—アミノ酸結合体の精製法（第一報）を少し改良し，より高度の精製品を得た。クロマト法により，このものは，酸性溶液でそれぞれ吸収の極大を，522 と 514mμ に有する二成分に分離できた。これらについて合成色素—アミノ酸結合体（第二報）と種々の性質を比較した結果，両者の間にはかなりの相違が認められた。天然の結合体についての推定構造は，チロシンを含むアミノ酸部分が色素のベンゼン核の 3 の位置に結合し，4 の位置の窒素は第一級および第二級アミンの混合であろうと推定される。

(文部省科学研究費による)

[GANN, 49, 97~103, June, 1958]

STUDY ON THE NATURE OF BINDING OF DIMETHYLAMINOAZOBENZENE WITH LIVER PROTEIN OF RATS FED THE CARCINOGEN

IV. AMOUNT AND NATURE OF THE POLAR DYES IN LIVER PROTEIN AFTER THE ADMINISTRATION OF AMINOAZO DYES WITH THE AID OF STOMACH TUBE

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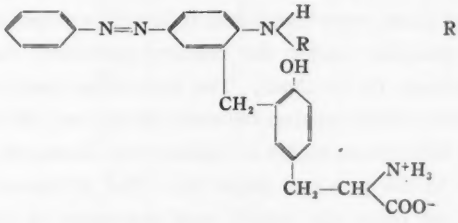
It was reported in the preceding paper that at least two types of the polar dyes were obtainable from the liver of rats fed dimethylaminoazobenzene (DAB) coated rice (0.06%). The following configurations were proposed for the chemical structures of the polar dyes to show the binding between the dye and proteins (1):



Recently Hultin (2) and also Miller et al. (3) have tried the 3′-Me-DAB administration with the aid of a stomach tube technique. Hultin observed that the dye bound protein was first found in the microsome fraction of the liver cells immediately after the administration of the dye. Whether the incorporation of the dye into a protein takes place in a stage of protein precursor or in the protein alreaey present in the microsome is a very interesting problem. Because of the high death rate among the experimental animals during the first several weeks of the feeding of DAB coated rice, the adoption of the stomach tube technique seemed very promising for studying the nature of polar dye formation in saving time as well as animals.

In this report, the amount of protein bound dyes and their chemical natures after the stomach tube administration of AB, MAB, DAB and AAT* (each 50mg) will be reported.

Methods and Procedures

1) **Treatment of the Animals:**

Albino male rats weighing 150g were administered with 50mg of aminoazo dyes (AB, MAB, DAB and AAT) dissolved in 2ml of propylene glycol directly into the stomachs with the aid of a stomach tube under ether anesthesia. The rats were fed rice coated with the respective aminoazo dye in 0.06% (for AB, MAB and DAB) (4) or in 0.1% (for AAT) (5) concentrations after the recovery from the anesthesia until they were sacrificed for the measurements of the protein bound dye.

2) **Preparation and Estimation of the Polar Dye:**

The defatted rat liver was subjected to the enzymic hydrolysis using the strong protease from **Streptomyces griseus** for 5 to 7 days at 35°C. The mixture was heated after the hydrolysis and was filtered to remove a small amount of insoluble fragments of the liver hydrolysate. The filtrate was once washed with benzene and then extracted with n-butanol. The butanol extracts were combined and evaporated under the reduced pressure. The residue was dissolved in 2N hydrochloric acid and ethyl acetate. The ethyl acetate layer was separated after shaking in a separating funnel. A small amount of polar dye can be recovered by extracting the ethyl acetate with a small volume of hydrochloric acid again. After the hydrochloric acid solutions were combined, followed by removing ethyl acetate from the solution by distilling under the reduced pressure, the solution was adjusted to a definite volume (10 to 15ml). The absorption spectra of the solution was measured in the wave length region between 600mμ and 380mμ.

The difference in the absorption at 510 to 520mμ was measured from the basal absorption as described in the previous paper (6). The difference was used for calculating the amount of polar dye which was expressed in term of a certain standard non-polar dye, for instance, DAB for the DAB polar dye. Since we do not know the actual molecular extinction coefficient of the respective polar dyes, the amounts thus calculated are just a measure of the yield, and cannot be compared in a strict quantitative sense among the different polar dyes.

Results and Discussions

The Amount of Protein Bound Polar Dyes:

The results on the time course change in the amount of protein bound dyes

* AB (Aminoazobenzene), MAB (N-Monomethylaminoazobenzene), DAB (loc. cit) AAT (ortho-Aminoazotoluene)

Table 1. The Amount of the Liver Protein Bound Dyes After the Intra-gastric Administration of Aminoazo Dyes.

Dye Administered	Time (hrs)	Liver Weight (g)	The amount of Protein Bounb Dye	
			per body (μg)	per 10g liver (μg)
DAB	6	5.6	* 0.8	1.5
	12	9.3	3.5	3.8
	24	9.3	10.4	11.5
	48	6.3	10.5	16.7
	57	7.7	22.2	28.8
	75	9.1	28.5	31.4
	81	9.1	19.5	21.4
	99	9.0	15.0	16.7
	125	9.6	13.9	14.4
	146	6.5	5.1	7.8
MAB	5	7.1	* 3.6	5.1
	12	8.4	5.3	6.2
	16	6.7	7.2	10.7
	27	7.0	7.5	10.7
	45.5	7.6	13.5	17.7
	50	7.3	9.3	12.7
	68	7.9	13.5	17.1
	76	6.1	5.9	9.6
	92	8.1	8.3	10.2
	118	6.7	6.2	9.3
	120	7.1	11.6	16.2
	138	9.1	7.6	8.4
AB	5	8.2	**3.5	4.3
	12	5.4	5.0	9.3
	25	8.5	2.6	3.1
	48	6.9	5.5	8.0
	49	8.8	2.7	3.1
	72	9.6	8.1	8.4
	75	9.8	8.5	8.7
	99	9.3	6.3	6.7
	99	10.2	4.0	3.9
	122	9.0	6.1	6.8
	123	8.2	7.1	8.7
	146	8.2	5.4	6.6
AAT	5	5.3	***5.6	10.6
	15	7.6	8.0	10.5
	25	6.4	8.0	12.5
	44	6.5	10.8	16.6
	51	4.8	10.5	21.9
	68	6.7	10.3	15.3
	93	7.4	13.6	18.3
	95	6.6	8.1	12.2
	113	6.0	7.7	12.8
	120	7.4	9.1	12.2
	144	7.6	10.1	13.3

* The amount was Calculated in terms of DAB as the standard. If 3-Me-MAB was employed as the standard, the values should be decreased by 12%. If 3-Me-AB was used, the values should be increased by 12%.

** The amount was calculated in terms of 3-Me-AB as the standard.

*** The amount was calculated in terms of N-Me-AAT as the standard.

after the intra-gastric administration of 50mg of dyes dissolved in 2ml of propylene glycol are listed in Table 1. It is very interesting to see that the maximum binding can be observed at about 70 hours after the administration of the dyes (see Fig. 1).

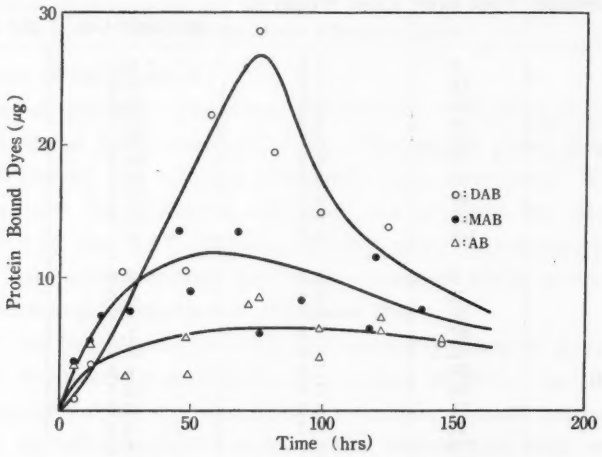


Fig. 1. The Time Course Change in the Amounts of Polar Dyes After the Intra-gastric Administration of Dyes

The almost similar situation has also been reported by Hultin (2) after the administration of 3′-Me-DAB (intra-gastric as well as intra-peritoneal) dissolved in olive oil.

The protein binding was the highest in the amount with DAB administration and the lowest with AB, MAB falling in between.

It seems very interesting to see how much the dye can be bound with liver protein. Can the protein binding go up as long as long as we put more dye into the experimental animals or is there a maximum capacity for the dye binding in the rat liver? This problem seems to be very important in knowing whether there is any specificity in the liver protein which is responsible fer the dye binding. If the binding takes place with non-specific liver proteins, the amount of protein bound dye would be expected to increase as the amount of DAB administered increases. Although the animals could not survive for a long time under the circumstances, we could obtain the enough data answering this problem. An experiment which was carried out for this purpose is as follows. Ten rats weighing about 150g were given 50mg of DAB dissolved in 2ml of propylene glycol with a stomach tube at every three days interval. One rat out of the group was sacrificed three days after the last administration of DAB and the liver protein bound dye was assayed as described above. Table 2 shows the result.

As shown in Table 2, it may be concluded, in spite of the considerable individual fluctuation, that the amount of the protein bound dye three dyas after the first

Table 2. The Protein bound Aminoazo Dyes from Rat Liver after the successive DAB Administration (Every three days interval, 50mg at one time)

No.	Liver Weight (g)	Protein Bound Dye* (μg)	Total DAB Administered (mg)	Days.
1	7.2	15.0	50	3
	9.1	28.5	50	3
2	8.2	18.2	100	6
3	6.5	12.0	150	9
4	7.1	26.8	200	12**

* Expressed in term of DAB ** Animals were exhausted at this time

administration of 50mg of DAB reaches the maximum combining capacity of the rat liver. The further administration of DAB does not increase the amount of the bound dye. This fact might be interpreted as showing that a specific protein or proteins are responsible for the dye binding. If we assume that one molecule of the dye is bound to a specific liver protein having about 60,000 molecular weight, then the amount of the specific proteins in the rat liver is approximately 4mg. The studies are being carried out in our laboratory to see the nature of the protein responsible for the binding.

The maximum amount of protein bound dye when DAB was fed under the ordinary conditions (0.06% in rice) is reached about at the 6th week and the value is only 4 to 8μg as expressed in term of DAB. It is apparent that the amount of the polar dye obtained from the intra-gastric administration of DAB is several times as much.

Nature of the Protein Bound Dyes:

It has been reported (1) that at least two types of polar dyes, having primary and secondary amino groups, were prepared from the rats fed rice containing 0.06% DAB. Relatively speaking, in this ordinary way of administration, the amount of the polar dye having amino group is greater than the polar dye having methyl amino group. However, as shown in this paper when a big amount of DAB is administered at one time, the relative situation in the amounts of the two types of polar dyes is reversed. The polar dye having methyl amino group (secondary amino) has been shown to be extremely large compared with the polar dye having the primary amino group (about 100 to 1). This has been confirmed by the chromatographic separation as well as by the nitrous acid test as described before (1).

The polar dye obtained from the rat liver administered 50mg of MAB was also a mixture of both types. But in this case the fraction of the primary amino type increased a bit, but still the polar dye having the secondary amino group was of majority. This finding was considered very peculiar at first.

If the bridge between the dye and the protein is made through the formalde-

hyde which is derived from the oxidative demethylation of N-methyl group of the dye, there is no difficulty in understanding that the two types of polar dyes can be derived from DAB, because DAB has two N-methyl groups which can be demethylated and turned into formaldehyde. At first, MAB was expected to give only one type of polar dye having the primary amino group because there is only one N-methyl group which is oxidatively liberated and is used for binding the remaining AB to the tyrosine residue of a certain protein.

This discrepancy between the expectation and the experimental results on the MAB polar dyes may be solved if we think of the fact that only a small fraction of the administered dye is combined chemically with proteins and the majority of the dye is metabolized, thus liberating a great amount of formaldehyde which can play a role in binding unattacked MAB as well as AB with proteins. There is also another possibility that MAB can be methylated into DAB through the endogeneous methylating system in the rat liver. Anyway the endogeneously supplied C_1 compound including formaldehyde is apparently playing a role in the binding of AB, because there is no N-methyl group in AB molecule.

Summary

1. The time course change in the amounts of protein bound dyes after 50mg of DAB, MAB, AB or AAT administeration directly into rats' stomachs was investigated and compared.

2. The protein bound dyes reached its maximum amount in about three days and gradually decreased after that. The maximum amount of protein bound dye was in the order: DAB > MAB > AB.

3. The polar dyes obtained from the DAB and MAB administrations consisted of two types of dyes: one having the secondary amino group (major part) and another having the primary amino group (minor part), while in the polar dye resulting from the AB administration only the presence of primary amino polar dye was shown.

4. The dye binding capacity of the rat liver reaches its maximum in three days after 50mg of DAB has been administered. Further administration of DAB does not seem to increase the dye binding.

5. C_1 compounds, probably formaldehyde, which can be produced in the oxidative metabolism of N-methyl groups of DAB and MAB as well as in the course of the basal liver metabolism was considered to play an important role in the binding between aminoazo dyes and certain liver proteins.

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要　旨

アミノアゾベンゼンの肝臓蛋白との結合に関する研究 第四報　種々のアミノアゾ色素をゾンデ投与した際の蛋白結合色素の量とその性質

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50mg の DAB, MAB, AB および AAT を 2cc のプロピレングリコールに溶かしたものを鼠の肝臓にゾンデで直接に注入したのち，肝臓蛋白質と化学的に化合した色素の量の時間的変化ならびにその結合の化学的様式について研究した。いずれの場合にも投与後 3 日目位で極大値に達し，それ以降は徐々に結合量は減少する。結合した色素量は DAB の場合が最も多く，MAB, AB の順に減少する。AAT の際にはほぼ AB に近い値を示すようである。DAB および MAB をゾンデで投与した場合は，白米に低濃度 (0.06%) に混ぜて長期間飼育した際に見られる最大結合量の数倍もの結合色素が短時日の中にみられ，かつ結合色素は第二級アミノ基恐らく $\left\{-N\begin{matrix}H\\CH_3\end{matrix}\right\}$ をもったものが大部分であることが特長的である。

DAB を 50mg 宛 3 日置きに投与した場合も結合色素量は第一回投与後特に増大する傾向は示されず，ほぼ一回の投与で肝臓の極限量に達するものと思われる。

種々な実験事実より，アミノアゾ色素に結合する蛋白質はかなり特異的なものであり，かつまた DAB や MAB の N-メチル基の代謝に由来する C_1 化合物 (恐らくフォルマリン) のみならず，基本的正常な代謝に由来する C_1 化合物もまた，アミノアゾ色素と蛋白質との結合に関与することが予想される。

(文部省科学研究費による)

[GANN, 49, 105~112; June, 1958]

PRELIMINARY INVESTIGATIONS ON THE PROTEIN BINDING OF ORTHO-AMINOAZOTOLUENE IN THE LIVER OF RATS FED THE CARCINOGEN

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(Cancer Institute, Japanese Foundation for Cancer Research Tokyo)

INTRODUCTION

Ortho-aminoazotoluene (AAT) is the first aminoazo dye which has been proved carcinogenic to rat liver (1, 2). The carcinogenicity of the aminoazo dyes was shown by Miller et al. to be related to its ability to form the protein bound dye in the experimental animals at the precancerous stage (3, 4, 5).

Miller et al. (3) suggested that at least one N-methyl group is necessary for being carcinogenic, for instance, DAB (dimethylaminoazobenzene) and MAB (monomethylaminoazobenzene) are almost equally potent carcinogens but AB (aminoazobenzene) shows only a weak carcinogenicity. The possibility that an oxidative intermediate of N-methyl group, probably formaldehyde, may be responsible for binding the dye with certain amino acid residues of liver proteins, had been suggested by Miller et al. (3), and the chemical nature of the DAB polar dye has been investigated by Kusama and Terayama (6, 7). Miller et al. have done a lots of works on the chemical binding of aminoazo dyes with liver proteins using various derivatives of aminoazobenzene, but there is no data concerning the protein binding of AAT, which has no N-methyl group but is rather a potent carcinogen. It has been suggested that very weakly carcinogenic AB is once methylated in the liver before it becomes carcinogenic (8). In order to investigate the chemical nature of the protein binding of the carcinogens, it seems interesting to study the protein binding of AAT in rat liver chiefly from the above reasons.

The results of our experiments still being carried out have revealed so far that AAT binds chemically with liver proteins. The defatted liver powder prepared from liver of rats fed AAT for various time intervals yielded a so-called polar dye after it was hydrolyzed by the *Streptomyces grisseus* protease (9). In this paper, the change in the amount of AAT polar dye in the time course of the feeding has been investigated and some preliminary investigations on the chemical characteristics of the partially purified polar dye will be reported. The synthetic

AAT-tyrosine polar dye which was obtained in a crystalline form from the alkaline hydrolyzate of AAT bound silk fibroin was compared with the natural AAT polar dye.

Experimental

Care of the Animals: Male albino rats were fed rice coated with AAT dissolved in olive oil. The content of AAT in rice was 0.1 per cent according to the original technique by Yoshida (1). The animals were fed ad libitum and once in a while some carrots were fed.

Preparation and Estimation of Polar Dye: The animals were sacrificed by decapitation and the liver was removed immediately and subjected to the homogenation in a Waring blendor. The homogenate (about 50%) was then heated in a boiling water bath after it was mixed with equal volume of methanol. The coagulated proteins were precipitated by centrifugation and washed successively with methanol, acetone and ether. The dried liver powder thus obtained was subjected to the enzymic hydrolysis using the purified protease from *Streptomyces griseus*. The pH of the reaction mixture was maintained at 7.0 in M/10 sodium acetate and occasionally checked and readjusted by sodium carbonate solution (2%). The mixture was incubated at 37°C for several days in the presence of toluene. At the end of the hydrolysis the most of the solid material disappeared into a brownish solution. The mixture was heated in a boiling water bath and filtered through cotton. The filtrate was washed with benzene to remove a trace of nonpolar dye. The polar dye was then extracted with n-butanol (thrice) and the butanol extracts were combined. The combined butanol extract was once washed with water and then filtered through cotton. The filtrate was then evaporated to dryness in a mild water bath under the reduced pressure. The residue was washed with 2N hydrochloric acid and ethyl acetate. After the two solutions were combined and shaked in a separation funnel, the pinkish aqueous layer was separated from the upper, yellow organic (ethyl acetate) layer. The organic layer was reextracted with a small amount of 2N hydrochloric acid and these extracts were combined. The ethyl acetate dissolved in the hydrochloric acid solution was driven off by the distilation under the reduced pressure. The absorption spectra of the solution was then measured with the aid of the Beckmann spectrophotometer (Model DU) at the wave length region

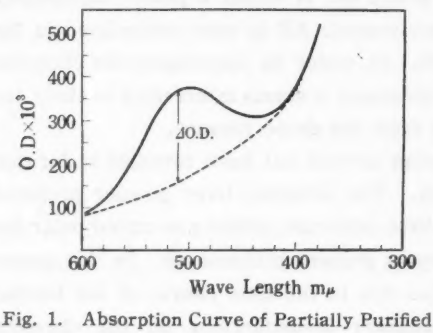


Fig. 1. Absorption Curve of Partially Purified AAT Polar Dye in 2N Hydrochloric Acid

from 600mμ to 350mμ.

The amount of the AAT polar dye was tentatively calculated from the absorption difference at 510mμ between the AAT polar dye solution and the basal solution. For the practical convenience, a value was tentatively taken as the basal absorption at 510mμ by extrapolating a part of the AAT polar dye absorption curve between 360mμ and 420mμ toward the longer wave length as shown in Fig. 1.

Preparation of the Synthetic AAT-Tyrosine Polar Dye: Synthetic AAT-tyrosine polar dye (a Mannich product) was prepared in a similar way as described for the synthetic AB-tyrosine polar dye (7). Two grams of silk was kneaded in 20ml of 50% sulfuric acid (v/v) until it becamep asty. As soon as it became pasty mass, 200ml of alcohol was added under vigorous stirring. The white precipitate was centrifuged and washed several times with alcohol, 4% sodium carbonate and water in succession. The precipitate was then resuspended in 100ml of water and 0.2 grams of AAT dissolved in 50ml of alcohol was mixed. 2ml of commercial formalin (35%) was added to the mixture and the whole reaction mixture was incubated at 35°C for three days.

The mixture was centrifuged and the precipitate was washed with alcohol to eliminate absorbed AAT. The yellowish AAT bound fibroin powder was put into a solution consisting of 4.5N NaOH 200ml and alcohol 50ml and was subjected to the hydrolysis for 11 hours under a reflux condenser on a boiling water bath. The hydrolyzate was washed with benzene to eliminate a trace of non-polar dye. n-Butanol was used to extract the polar dye from the hydrolysate. The butanol extracts were combined and washed with water. The butanol was evaporated under reduced pressure and yellow residue was dissolved in 2N hydrochloric acid. The pink solution was submitted to the column chromatography using IRC-50 (H-type). The absorbed polar dye at the top of the column was washed with 2N hydrochloric acid, water and 30% alcohol and finally eluted with 60% alcohol. The eluate was fractionated into three different parts. Fraction II, which was a main fraction, was submitted to the alumina (Brockman, HCl pretreated) chromatography. After washed with 60% alcohol, it was eluted with water. The water eluate separated fine needle crystalls upon standing (Fig. 2). The pH of the mother liquid was 4.0.

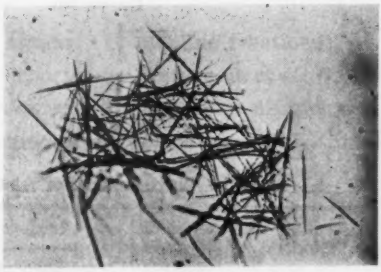


Fig. 2. Crystals of the Synthetic AAT-Tyrosine Polar Dye

The crystalls showed a strong positive reaction to the Folin-Ciocalteau's phenol reagent and seems to be a AAT-tyrosine polar dye.

Absorption Spectra of AAT, N-Methyl AAT, Synthetic AAT-tyrosine and

Natural partially purified AAT polar dye: Since the naturally obtainable AAT polar dye is very labile compared with the natural DAB polar dye, the satisfactory purification of the natural AAT polar dye has not been succeded yet. The absorption spectra of AAT, N-meteyl AAT and synthetic AAT-tyrosine polar dye are shown in Fig. 3.

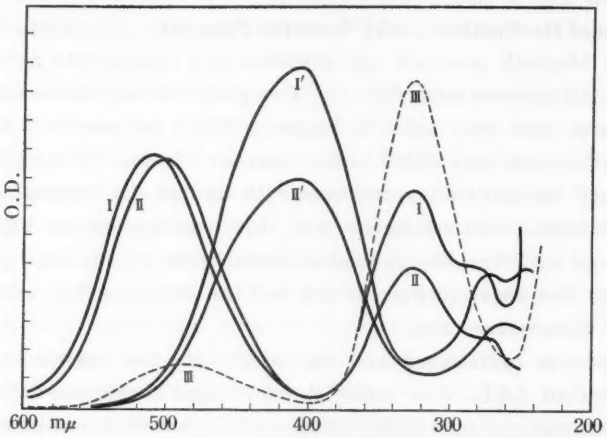


Fig. 3. Absorption Curves of AAT, N-Me-AAT and Synthetic AAT-Tyr Polar Dye

Curve No.	Substance	Solvent
I	AAT-Tyr P.D.	N HCl, 30% alcohol
I′	AAT-Tyr P.D.	30% alcohol
II	N-Me-AAT	N HCl, 30% alcohol
II′	N-Me-AAT	30% alcohol
III	AAT	N HCl, 30% alcohol

Table 1. Wave Length of Absorption Maxima of AAT and Its Derivatives

Samples	Acidic			Alkaline Absorption Max.
	Absorption Max.		Intensity Ratio (DQ to D_R)	
	Q	R		
AAT	490mμ	326mμ	0.14	407mμ
N-Me.-AAT	503	328	1.75	407
Synth. AAT-Tyr P.D.	510	330	1.16	406
Natural AAT-P.D.	Ca. 510	?	—	—

As shown in the above table, the absorption spectra of the natural polar dye appears to be similar to the synthetic AAT-tyrosine polar dye.

Stability of Polar Dyes: As a measure of testing the stability of dyes against acid treatment, the change after refluxing dyes in 1N sulfuric acid for 2 to 3 hours

was compared. As described in the preceding paper (10), the natural DAB polar dye was shown rather stable and did not give rise to the formation of considerable amount of nonpolar dye after the acid treatment, while the synthetic AB-tyrosine polar dye (a Mannich product) was decomposed almost quantitatively into AB and a tyrosine derivative.

The polar dyes containing AAT, either natural or synthetic, were shown very labile against the acid treatment. The synthetic AAT-tyrosine polar dye gave nonpolar dye, the absorption spectra of which is almost similar to that of AAT (Fig. 2). The yield of nonpolar dye was about 100% from the original synthetic polar dye. The natural AAT polar dye was also very unstable in acid media. The absorption at 510mμ and its vicinity decreases rapidly and the pink color tone is greatly reduced and often the solution becomes darker. The chromatographic purification using IRC-50 (H-type) in hydrochloric acid which was efficiently used for the purification of DAB polar dye was shown inadequate for the natural AAT polar dye due to its instability.

In contrary to the reaction against acid, the polar dyes are rather stable against alkali. As a matter of fact, the synthetic AAT-tyrosine polar dye was prepared after hydrolysing AAT bound silk fibroin with alcoholic alkali. In this case only a neglisible amount of nonpolar dye was obtained by extracting the hydrolysate with benzene. The crystalline AAT-tyrosine polar dye was also submitted to 1N NaOH hydrolysis for three hours on a water bath without causing a serious damage of the dye.

The natural AAT polar dye was also stable against the alkaline treatment.

Reactions with Nitrous Acid: It has been reported by Terayama et al. (7, 10) that the aminoazo dye having the primary amino group reacts with nitrous acid irreversibly, the one having the secondary amino group reacts reversibly, form

Table 2. Some Qualitative Tests for the Polar Dyes

Samples	Reaction with Nitrous Acid*		Reaction with Folin-Ciocalteau's phenol reagent
	At room temp.	Upon heating	
AAT	light yellow	light yellow	negative
N-Me-AAT	colorless	pink	negative
Synth. AAT-Tyr P.D.	colorless	yellow	positive
Natural AAT P.D.	yellow	yellow (darker)	positive

*A few drops of 0.1% $NaNO_2$ were added to the dye dissolved in hydrochloric acid.

ing a N-nitroso compound which can be easily decomposed to the original secondary amino group, and the one having the tertiary amino group does not react easily with nitrous acid in low concentrations. This reaction usually is accompanied by the typical color change of the aminoazo dye and can be used

as an identification method for the chemical characteristic of the amino groups of the dyes. Table 2 shows the reactions of the dyes with nitrous acid as well as with the Folin-Ciocalteau's phenol reagent.

The color changed from pink to the ones described in the column in Table 2. The presence of alcohol in the testing medium retards the decoloration of the dyes having the primary or the secondary amino groups due to the reaction with nitrous acid, but accelerates the reappearance of the pink color (decomposition of N-nitroso compound) with the dye having the secondary amino group.

The Time Course Change in the Amount of the Polar Dye: Three experimental animals were sacrificed at ten to twenty days interval and the amount of the polar dye from the liver hydrolysate was determined as described above. The amount of the polar dye per ten grams of fresh liver was plotted against the period of

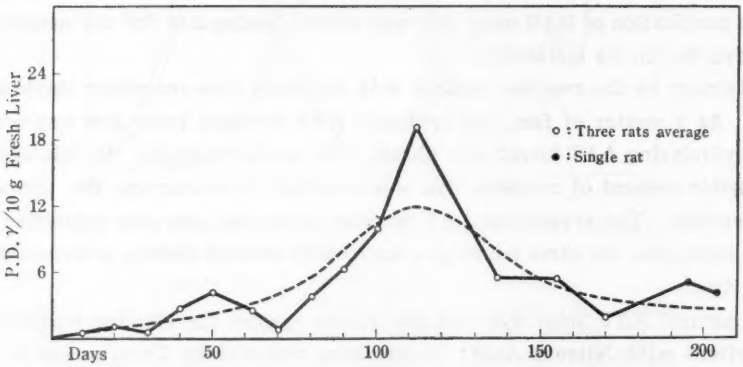


Fig. 4. Time Course Change in the Amount of the Protein Bound Dye in the Liver of Rats fed AAT (Expressed in terms of N-Me-AAT)

feeding of the diet (rice containing 0.1% AAT) as illustrated in Fig. 4. The change in the amount of the AAT polar dye thus observed showed some what irregular behavior due to some unknown reasons, but generally speaking, the amount of the protein bound dye increased in the time course of feeding, probably up to the third or the fourth month of feeding and then dropped down gradually before the hepatoma appeared. With our case, the first observation of the cirrhosis due to the administration of AAT was observed with one of the experimental animals at the 204th day of the feeding.

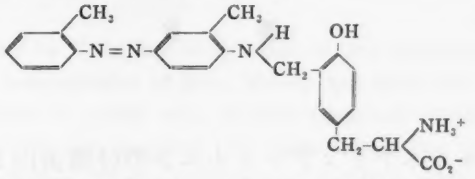
Results and Discussion

From the results of our preliminary experiments on the polar dye from rat liver fed AAT, it is clear that the oral administration of AAT gives rise to the accumulation of the protein bound dye in the rats liver up to the early few months and

then the amount gradually decreases.

As to the chemical structure of the natural polar dye, we can not say much at the present time, even though the chemical nature of the natural polar dye resembles much with that of the synthetic AAT-tyrosine polar dye. The further purification of the natural polar dye is necessary before the definite answer for the structure of the polar dye can be drawn.

The synthetic AAT-tyrosine polar dye is considered to have an ordinary structure assumed for the Mannich reaction product.



The $-NH-CH_2-$ bond in the synthetic polar dye is rather weak and can be easily broken by the acid treatment, thus liberating AAT and the tyrosine derivative.

Summary

1. Accumulation of the protein bound dye in the liver of rats fed AAT (ortho-aminoazobenzene) has been investigated.

2. Preliminary investigations have revealed that the polar dye from the rat liver fed AAT has some different chemical characteristic from the DAB polar dyes, especially in the stability against the acid treatment.

Acknowledgements

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要　旨

オルトアミノアゾトルエンの肝臓蛋白との結合に関する予備的研究

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AAT (オルトアミノアゾトルエン) を数カ月間白米に 0.1% の割合に混じた飼料で飼育した際ラッテ肝臓中の蛋白質と化学的に結合する色素の量の時間的変化を調査したところ，3カ月乃至4ケ月附近に極大値が存在することが分った。肝臓内で代謝されてフォルマリンを与えるようなN-メチル基をもたない AAT がかように蛋白質と結合することは興味深い。

絹フィブロイン中のチロシン残基にマンニッヒ反応を行わして AAT を結合させたのち，これをアルコール性アルカリで加水分解したのちAAT-チロシン結合体を結晶状にうることができた。

この合成 AAT-ポーラーダイと天然ポーラーダイ (部分的精製) について，若干の化学的性質の比較を行った。

(文部省科学研究費による)

[GANN, 49, 113~123; June, 1958]

LIPID PEROXIDE FORMATION AND ZINC IN NORMAL AND TUMOR TISSUES

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It has been reported by Kohn and Liversedge (1) and Bernheim, Bernheim, and Wilbur (2) that when homogenates of liver, kidney and brain are aerobically incubated and then allowed to react with thiobarbituric acid (TAB) a pink color is produced in the solution; this suggests that these tissues can produce lipid peroxides on aerobic incubation. That certain unsaturated fatty acids and phospholipids also produce these TAB reacting substances upon either ultraviolet irradiation or aerobic incubation was reported by Bernheim *et al.* (2) and Wilbur, Bernheim and Shapiro (3). Lipid peroxides formed by exposing methyl linolenate to ultraviolet radiation were reported to inhibit the growth of *Escherichia coli* (4) and the division of the eggs of the annelid *Chaetopterus* (5). Also, it was reported that peroxides are not formed in bone marrow where cell division is actively taking place (6). Regenerating rat liver shows minimal peroxide value 48 hours after partial hepatectomy when mitosis is maximal (7). These findings suggest that oxidized lipids inhibit cell division and that dividing cells contain some antioxidants. In this connection the work of Shuster (6) is extremely interesting. He showed that homogenates of Ehrlich ascites tumor produced no TBA reacting substance whatsoever on incubation or irradiation.

In the present study, the conditions determining the formation of TBA reacting substances (presumably lipid peroxides) were examined in normal and tumor tissues. It was found that tumors showed very low peroxide values as compared with normal tissues and that when ethylenediaminetetraacetic acid (EDTA) was added to the reaction mixture, the lipid peroxides were no longer formed, suggesting that some metals are involved in the formation of the peroxide. It turned out that of the metals examined, zinc alone was effective in restoring the peroxide formation in EDTA-treated systems.

Material and Methods

Male adult albino rats were used as material. Normal livers, primary hepatomas, transplanted Umeda-fibrosarcomas (rhodamine sarcoma), and the livers of fibrosarcoma-bearing rats were respectively examined. Hepatoma was obtained by the

usual butter yellow feeding and Umeda-fibrosarcoma by transplanting subcutaneously every 3-4 weeks. The rat was killed by decapitation and exsanguination, and the tissues were removed and washed in a 0.9 per cent NaCl solution. 1.0g of tissue was then homogenized in 15ml of 1/15M phosphate buffer solution, pH 7.3. Since 1.0ml of the buffer solution was added to 1.0ml of this original homogenate, the final concentration of homogenate thus obtained was 1 : 30. In experimental series 1.0ml of this buffer solution may contain various reagents, such as ascorbic acid, disodium salt of ethylenediaminetetraacetic acid (EDTA), $CoSO_4$, $CuSO_4$, $CuCl_2$, $FeCl_3$, $MnSO_4$, $MnCl_2$, $NiSO_4$, $ZnSO_4$, $ZnCl_2$, 2, 4-dinitrophenol (DNP), NaN_3, NaF and Na_2HAsO_4, these were respectively added to 1.0ml of the original homogenate. Samples thus obtained were incubated aerobically at 38°C with shaking for 2 hours, and at the end of the incubation period 2.0ml of 20 per cent trichloroacetic acid and 4.0ml of 0.67 per cent thiobarbituric acid (TBA) were added, and then the mixture was placed in a boiling water bath for 15 minutes. The pink color appeared in the course of boiling. After cooling, another 2.0ml of 20 per cent trichloroacetic acid and 6.0ml of deionized water were added, centrifuged, and the pink color in the supernatant was measured in a Hitachi photometer with a No. 50 filter. The TBA values (that is, the optical density values for TBA reacted substances) were thus obtained, and the results represented in this paper are shown as $(-\text{Log } T)\times 100$. Although it was not confirmed in the present study that the TBA reacting substances formed on incubation are identical with lipid peroxides, they will tentatively be called lipid peroxides in what follows.

Results

Preliminary experiments. The TBA values expressed as $(-\text{Log } T)\times 100$ for normal liver at various pHs are shown in Figure 1. The highest TBA value at pH 6.0 indicates that the lipid peroxides are formed exceedingly at this pH. Although the pH optimum of peroxide formation was found to be at pH 6.0, the following experiments were all carried out at the physiological condition, pH 7.3. The relationship between lipid peroxide formation and tissue concentration was then studied in normal liver, it was found that the peroxide formation was proportional to the tissue concentration from 1 : 120 to 1 : 15 homogenate. Further, the effect of incubation time on the lipid peroxide formation in normal liver was examined, and, as shown in Figure 2, it was found that the rate of peroxide formation is almost proportional to the incubation time within 120 minutes.

Experiment 1. The activities of lipid peroxide formation in various tissues, such as normal livers, livers of fibrosarcoma-bearing rat, hepatomas, and fibrosarcomas were comparatively examined with and without 0.2mg/ml of ascorbic acid (Table 1). The average TBA value was 15.6 in normal liver, 9.1 in the liver of tumor-bearing rat,

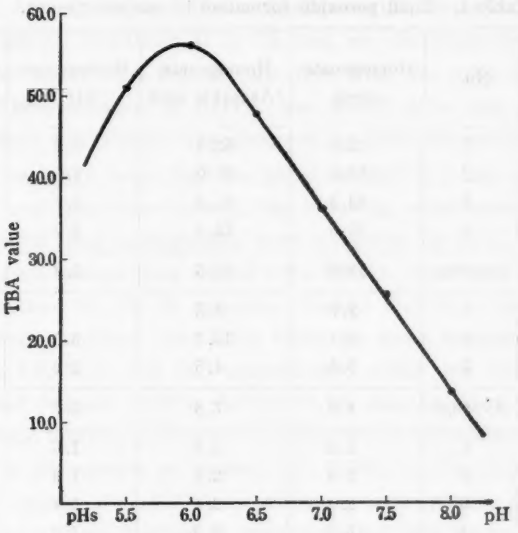

Fig. 1. pH-activity curve of lipid peroxide formation in normal rat liver.

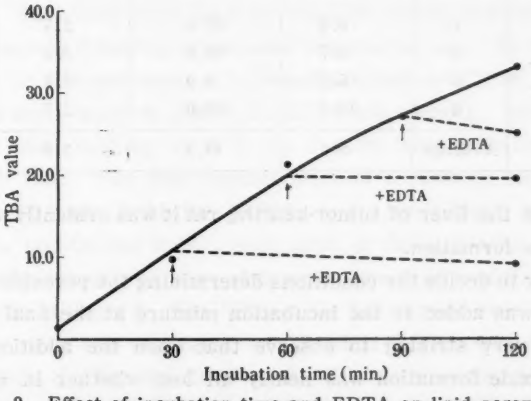

Fig. 2. Effect of incubation time and EDTA on lipid peroxide formation in normal rat liver.

4.9 in hepatoma, and 2.9 in fibrosarcoma. It is thus obvious that the activity of lipid peroxide formation is considerably decreased in the case of the liver of tumor-bearing rat and greatly decreased in hepatoma and fibrosarcoma. In the presence of ascorbic acid, as shown in Table 1, the average TBA value became 56.6 in normal liver, 44.4 in the liver of tumor-bearing rat, 7.8 in hepatoma, and 2.6 in fibrosarcoma. It is to be noted that ascorbic acid could not accelerate the production of lipid peroxide in the case of tumor tissues, whereas in the case of

Table 1. Lipid peroxide formation in various tissues.

	No.	Homogenate alone	Homogenate +Ascorbic acid	Homogenate +EDTA	Homogenate +Ascorbic acid +EDTA
Normal liver	1	13.0	52.5	2.5	2.5
	2	13.0	51.0	1.5	2.1
	3	13.8	51.0	1.7	2.1
	4	22.5	72.0	3.0	2.6
	Average	15.6	56.6	2.2	2.3
Hepatoma	1	2.7	3.8		
	2	6.4	15.1	3.2	3.1
	3	5.6	4.5	2.2	2.6
	Average	4.9	7.8	2.7	2.8
Fibrosarcoma	1	1.8	2.2	1.5	2.1
	2	1.8	2.4	1.3	2.1
	3	2.4	2.4	1.4	1.7
	4	5.4	3.3	1.2	1.6
	Average	2.9	2.6	1.4	1.9
Liver of fibrosarcoma-bearing animal	1	8.3	61.0	2.5	3.5
	2	8.7	50.5	3.2	4.6
	3	5.2	9.0	3.3	3.9
	4	14.3	57.0	2.7	3.2
	Average	9.1	44.4	2.9	3.8

normal liver and the liver of tumor-bearing rat it was evidently acting by enhancing the peroxide formation.

Next, in order to decide the conditions determining the peroxide formation in the tissues, EDTA was added to the incubation mixture at the final concentration of 10^{-3}M. It was very striking to observe that upon the addition of EDTA, the activity of peroxide formation was nearly all lost whether in normal or tumor tissues. The TBA value for normal liver was 2.2 on an average, 2.9 for the liver of tumor-bearing rat, 2.7 for hepatoma, and 1.4 for fibrosarcoma (See Table 1). As also shown in Table 1, the TBA value, in the presence of both EDTA and ascorbic acid, is 2.3 in normal liver, 3.8 in the liver of tumor-bearing rat, 2.8 in hepatoma, and 1.9 in fibrosarcoma. It appears therefore that in all tissues the reduced activity of peroxide formation by EDTA could not be restored to normal even in the presence of ascorbic acid. These results suggest that some metals are essential for the production of the peroxides in various tissues.

Experiment 2. The inhibitory effect of EDTA was further studied using various

concentrations. A complete inhibition of lipid peroxide formation was obtained at 10^{-3} and 10^{-4}M, partial inhibition at 10^{-5}M, and no inhibition whatever below 10^{-6}. It was also found that EDTA did not interfere with the color formation of lipid peroxide with TBA, because, if EDTA was added after the tissues had been incubated, no effect whatsoever was observed on color formation.

Furthermore, to make sure that the effect of EDTA is not due to the decomposition of lipid peroxide once formed, the following experiment was carried out; 3 samples of normal liver homogenate were incubated at 38°C, and after 30, 60, and 90 minutes each sample was mixed with EDTA, and these were continuously incubated for another 90, 60, 30 minutes. Hence the total incubation time was 120 minutes in each sample. The TBA value for these samples, as represented in Figure 2, was 8.8, 19.6, and 24.5 respectively, while the values for another 3 samples incubated without EDTA for 30, 60, 90 minutes were 9.2, 21.4, and 26.8 respectively. Since there is practically no decrease in the amount of the peroxide at a given time if the sample is treated with EDTA during the course of incubation, it does not appear that EDTA is acting by decomposing the lipid peroxide once formed. From these results, it was concluded that some metals are acting as catalyst in the processes of the formation of lipid peroxides.

Experiment 3. To decide what kind of metal or metals is indispensable for the peroxide formation, the following experiment was carried out. To the homogenate of normal liver or fibrosarcoma, a buffered solution of EDTA plus a buffered solution of metal salts, such as $CoSO_4$, $CuSO_4$, $FeCl_3$, $MnSO_4$, $NiSO_4$, and $ZnSO_4$ were added. and after shaking at 38°C for 2 hours lipid peroxides formed were measured in each case. The final concentration of incubation mixture was 1 : 30 for homogenate, 10^{-3} or 10^{-4}M for EDTA, and 1/200-1/1000M for metal salts. Instead of $CuSO_4$, $MnSO_4$ and $ZnSO_4$, such salts as $CuCl_2$, $MnCl_2$ and $ZnCl_2$ were also tested, and similar results were obtained. The results of the experiments of this series are given in Table 2. The TBA value for normal liver was 21.7 on the average, but this value was decreased to 2.3 when EDTA was added. Further addition of either Co, Cu, Fe, Mn or Ni to the EDTA-containing sample had little effect on the TBA values once reduced, the values being 2.4, 5.5, 4.5, 3.8, and 2.7, respectively. However, it is to be noted, when Zn was added, the TBA value was remarkably restored (18.3). In the case of fibrosarcoma, the TBA value was about 9.2 without EDTA, but 1.7 with EDTA. If Co, Cu, Fe, Mn or Ni was added to the EDTA-containing incubation medium there was no appreciable increase in the TBA value, although a slight increase was observed in the case of Fe. In the case of Zn addition, however, an almost complete restoration was obtained. The recovery was greater in the case of fibrosarcoma than in normal liver, and it is to be noted that the TBA value for fibrosarcoma homogenate containing both

Table 2. Effect of metals on lipid peroxide formation with EDTA

	No.	EDTA conc. (M)	Metal conc. (M)	Homogenate alone	Homogenate+EDTA						
					Without metal	Co	Cu	Fe	Mn	Ni	Zn
Normal liver	1	1/1000	1/200	13.3	1.7	1.7	7.1	2.8	3.3	1.7	11.3
	2	1/1000	1/200	23·2	2.6	2.6	4.4	3.7	4.6	2.6	16.2
	3	1/1000	1/200	28.7	2.6	2.7	8.3	4.3	4.9	2.1	23.3
	4	1/1000	1/400	28.7	2.6	2.7	8.8	4.3	3.7	2.2	26.2
	5	1/1000	1/400	17.7	2.0	2.3	2.3	5.8	4.0	2.5	17.0
	6	1/10000	1/1000	18.6	2.3	2.3	2.3	6.2	2.0	5.0	15.5
	Average			21.7	2.3	2.4	5.5	4.5	3.8	2.7	18.3
Fibrosarcoma	1	1/1000	1/400	5.4	1.2	1.6	1.2	4.9	2.6	1.6	9.3
	2	1/1000	1/400	10.4	2.1	1.9	2.7	5.7	2.5	1.9	9.0
	3	1/1000	1/400	11.8	1.8	1.7	1.8	6.5	2.6	1.9	14.5
	Average			9.2	1.7	1.7	1.9	5.7	2.6	1.8	10.9

EDTA and Zn exceeded that for homogenate alone. Therefore, it may be concluded that among the metals examined, zinc is the only metal which can catalyse the formation of lipid peroxides in normal and tumor tissues.

Experiment 4. Then, the effect of various metals was examined upon the homogenates without the addition of EDTA. As shown in Table 3, it was found that the TBA value for normal liver homogenate was about 18.2 without metal, 2.6 with Co, 2.6 with Cu, 30.6 with Fe, 3.3 with Mn, 6.4 with Ni, and 17.4 with Zn. The value for fibrosarcoma homogenate alone was 11.1, and 2.6, 3.4, 23.6, 3.5, 2.6, and 8.9 in the presence of Co, Cu, Fe, Mn, Ni, and Zn, respectively. The lipid peroxide formation appeared to be inhibited by Co, Cu, Mn, and Ni both in normal liver and fibrosarcoma homogenates. Zinc was also found to be slightly inhibitory in the case of normal liver and fibrosarcoma. It was very interesting

Table 3. Effect of metals on lipid peroxide formation without EDTA

	No.	Metal conc. (M)	Homogenate						
			Without metal	Co	Cu	Fe	Mn	Ni	Zn
Normal liver	1	1/400	17.7	2.7	2.6	30.1	3.9	2.7	17.7
	2	1/1000	18.6	2.5	2.5	31.0	2.6	10.1	17.0
	Average		18.2	2.6	2.6	30.6	3.3	6.4	17.4
Fibrosarcoma	1	1/400	10.4	2.4	3.8	22.7	3.2	2.4	7.7
	2	1/400	11.8	2.7	2.9	24.4	3.7	2.8	9.9
	Average		11.1	2.6	3.4	23.6	3.5	2.6	8.9

however that iron produced a marked acceleration when added alone.

The effect of zinc upon fibrosarcoma homogenate alone seems to be inconsistent with the results previously described that the TBA value for fibrosarcoma homogenate containing both EDTA and Zn is greater than the value for homogenate alone (cf. Table 2) But, it is possible to assume that the final concentration of zinc in

Table 4. Effect of zinc at various concentrations on lipid peroxide formation without EDTA

	No.	Homogenate alone	Homogenate+Zn				
			1/500M	1/1000M	1/2000M	1/4000M	1/8000M
Normal liver	1	27.0	17.5	17.8	18.3	19.7	20.9
	2	22.6	13.5	13.8	15.1	16.0	17.0
	3	28.7	15.9	16.9	18.3	20.2	21.4
	Average	26.4	15.6	16.2	17.2	18.6	19.8
Fibrosarcoma	1	6.7	7.1	8.6	9.6	9.8	10.0
	2	3.3	3.4	4.5	4.9	5.7	5.7
	3	8.8	6.2	7.0	7.3	8.8	9.8
	Average	6.3	5.6	6.7	7.3	8.1	8.5

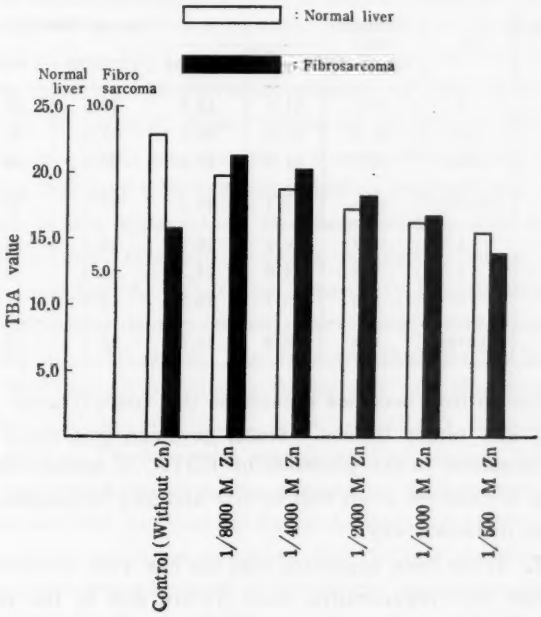

Fig. 3. Effect of zinc at various concentrations on lipid peroxide formation without EDTA.

the homogenate plus Zn (in Table 3) was much higher than that of homogenate plus EDTA plus Zn, because in the latter case excess of EDTA had been added and that excess of zinc may interfer with peroxide formation. To test this possibility the following experiment was performed.

Experiment 5. The effect of zinc at various concentrations was studied and the results are given in Table 4. As shown in the Table, the inhibitory effect of zinc becomes less as the concentration is lowered and when the concentration is 1/8000M, the inhibition of lipid peroxide formation is very slight in the case of normal liver. However, in the case of fibrosarcoma, the peroxide formation appears to be accelerated by zinc especially at lower concentrations. At any rate, it is of special interest that zinc can enhance the production of lipid peroxide in the tumor even when it is added alone. The results are summarized in Figure 3.

Experiment 6. It has been shown that whereas iron is not able to restore the EDTA-reduced activity of peroxide formation, it can greatly accelerate the activity when added alone. The effect of iron at various concentrations was therefore examined. As shown in Table 5, it was found that in contrast to the case of zinc

Table 5. Effect of iron at various concentrations on lipid peroxide formation without EDTA

	No.	Homogenate alone	Homogenate+Fe				
			1/500M	1/1000M	1/2000M	1/4000M	1/8000M
Normal liver	1	27.0	44.5	42.6	39.5	37.4	36.1
	2	22.6	37.3	36.7	34.2	31.7	28.8
	3	23.7	44.5	40.6	37.6	36.0	34.1
	Average	26.4	42.1	40.0	37.1	35.0	33.0
Fibrosarcoma	1	6.7	19.2	18.8	18.2	15.9	13.8
	2	3.3	14.9	14.3	14.1	13.3	11.5
	3	8.8	13.7	13.3	12.9	11.4	11.4
	Average	6.3	15.9	15.5	15.1	13.5	12.3

the activation due to iron becomes greater as the concentration becomes higher both in normal and tumor tissues. Since, however, iron could not activate the lipid peroxide formation in the presence of EDTA, it seems that the activation induced by iron is different from that by zinc and may be coupled with some other systems in some unknown way.

Experiment 7. It has been suggested that the low TBA values in tumor tissues (6), bone marrow and regenerating liver (7) are due to the presence of some antioxidants in these actively proliferating tissues. However, when homogenates of fibrosarcoma were added to liver homogenates, no inhibition of peroxide for-

mation was found. Therefore, it is not likely that reduced activity of peroxide formation in this sarcoma is due to antioxidant activity.

Experiment 8. Lastly, effects of 2, 4-dinitrophenol (DNP), NaN_3, NaF, and Na_2HAsO_4 upon the peroxide formation were examined. It was found that the TBA values for control normal liver homogenate was 27.0, and the value in the presence of DNP (10^{-4}M), NaN_3 (10^{-3}M), NaF (10^{-3}M), and Na_2HAsO_4 (10^{-3}M) is 24.0, 18.8, 27.2, and 27.0, respectively, it may be said therefore that the production of lipid peroxide in normal liver is slightly inhibited only by NaN_3, other reagents being without effect.

DISCUSSION

In the present study, it has been found that hepatoma and fibrosarcoma show minimal peroxide values, and this result is in accord with the result reported by Shuster (6) that Ehrlich ascites tumor produce no peroxide although it contains more linolenic acid than normal liver. Shuster suggested that the negative TBA test indicates an increased antioxidant activity in the tumor. According to Wolfson, Wilbur and Bernheim (7), preparation of bone marrow does not form peroxide on incubation although it contains the requisite lipids and, moreover, bone marrow suspensions added to liver homogenate inhibit peroxide formation in the latter. They also suggested the presence of antioxidant in regenerating rat liver in which active mitosis is taking place and TBA values are low. In the present study, however, it was found that fibrosarcoma contains no antioxidant. The main point of importance in the present paper is that on addition of EDTA the production of peroxide with or without ascorbic acid is completely inhibited whether in normal or tumor tissues and that it is again restored to original level by the addition of zinc. Although it was reported by Bernheim, Wilbur and Fitzgerald (8) that cyanide had little effect, this may be due to weaker action of cyanide as metal-binding agent than EDTA. That zinc appears to be indispensable for peroxide formation in normal and tumor tissues is very interesting, since Fujii (9, 10, 11, 12), in a series of publications, has reported that zinc is found in high concentration in the nucleoli of various forms and that the chromosomes and the spindle become dithizone-positive on entering mitosis in sea-urchin eggs and staminal hair cells of *Tradescantia*, suggesting that zinc is involved in mitosis. Fujii (13) also has found that the zinc content of rat hepatoma is always higher than that of normal liver and that the zinc in hepatoma is hardly freed or extracted from its firmly bound state, as compared with the case of normal tissues. Therefore, it appears that certain changes in the nature of the zinc-protein linkage are, in part, responsible for the neoplastic transformation of cells and that the low TBA values in tumor tissues are associated with these changes.

On the other hand, it has been suggested that lipid peroxide is acting by controlling mitosis (4, 5, 6, 7). It seems therefore extremely interesting that the TBA values are very low in tumor tissues and that zinc is involved in the lipid peroxide formation. As regards the effect of iron further studies are now in progress.

Summary

1. The production of thiobarbituric acid (TBA) reacting substances (lipid peroxides) was examined in normal rat liver, liver of fibrosarcoma-bearing rat, rat primary hepatoma, and transplanted fibrosarcoma.

2. The capability of such peroxide formation is considerably decreased in livers of tumor-bearing animal, and almost disappeared in tumor tissues.

3. Peroxide formation is accelerated by ascorbic acid and completely inhibited by EDTA. In the presence of EDTA, activating effect of ascorbic acid is no longer observed. This reduced activity by EDTA can be restored only by the addition of zinc.

4. Zinc is the only metal which participates by itself in such peroxide formation, although iron appears to accelerate this reaction in combination with some unknown factor when added to intact homogenates.

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要　旨

癌組織における lipid peroxide の形成と亜鉛

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正常白ネズミの肝臓，肉腫をもった白ネズミの肝臓，原発肝癌，移植肉腫について lipid peroxide の形成能力を比較したところ，担癌動物の肝臓では lipid peroxide の形成能力がかなり低下しており，さらに癌組織ではほとんど消失してしまっている。このような lipid peroxide の形成は正常肝臓と担癌動物の肝臓ではアスコルビン酸で促進されるが，癌組織では促進されない。しかしいずれの場合も EDTA によって完全に抑制される。なお EDTA が存在しているとアスコルビン酸を加えてもその促進作用があらわれない。しかし EDTA による抑制は亜鉛を加えることによって消失してしまうから，亜鉛は lipid peroxide の形成に重要な役割をはたしているものとおもわれる。コバルト，銅，マンガン，ニッケル，鉄にはこのような作用がない。ただ EDTA の存在していないときにかぎり，lipid peroxide の形成は鉄によって著しく高められることから，鉄は未知の因子との協同作用でこのような促進効果をもたらすものと考えられる。

（文部省科学研究費による）

[GANN, 49, 125~136; June, 1958]

STUDIES ON TRYPTOPHAN PEROXIDASE IN THE LIVER OF TUMOR-BEARING ANIMALS.

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Tryptophan is oxidized to formylkynurenine by two step oxidation of peroxidase-oxidase system in the liver of animals (1). And tryptophan peroxidase (TPO) appears to be unique among mammalian enzymes in that its activity can be increased several folds *in vivo* by the administration of its substrate, tryptophan (2—5). The activity of TPO can be also increased to a less extent by administering substances, such as cortisone or histidine, which cause the eventual release of adrenal hormones in the treated animals (3, 6—10). This adaptive increment of TPO activity seems to be induced by the synthesis of a new enzyme. The synthesis of the new enzyme during the adaptation was demonstrated by Lee *et al.* and Dietrich; the increase of TPO activity in animals given tryptophan was prevented by ethionine and this inhibiting effect of ethionine was overcome by large doses of methionine (11, 12). Further evidence of the new enzyme formation was established by Greig and Carter; the selective incorporation of labelled amino acid into the partially purified preparation of TPO occurred after the adaptation (13).

Norberg and Greenberg showed that a tumor exerts a systematic effect on the host and induces an alteration in the normal protein metabolism (14). On the other hand, TPO seemed to be ferric enzyme, as it is inhibited by the addition of azide, cyanide, sulfide or carbon monoxide (15). Many investigations have been made on the reduction in activity of the ferric enzymes such as catalase and cytochromes in the liver of tumor-bearing animals. According to Nakahara and his co-workers, this depression in the activity of these enzymes may be caused by the disturbance in iron metabolism in tumor-bearing animals (16—18). Since the adaptation of TPO seems to occure by the new enzyme formation in a relatively short period, the study on TPO of the liver in tumor-bearing animals may be expected to yield some informations on the protein and iron metabolism as affected by the tumor growth.

In this report are presented the results of the TPO activities in the liver of tumor-bearing animals treated with tryptophan or histidine.

1. Studies on the liver of mice bearing Ehlrich ascites carcinoma. Female adult mice of dd strain, weighing 18 to 25g, were used in this experiment. Mice

were divided in the following four groups. Mice of the first group, were inoculated with Ehrlich ascites carcinoma intraperitoneally (Tumor-bearing (I)). About 8 to 10 days after the inoculation, these animals were sacrificed for the experiment. In the second group, each mouse received the inoculation of the Ehrlich ascites carcinoma subcutaneously (Tumor-bearing (S)) and was then allowed to grow the solid tumor for at least 15 days. When the tumor weight surpassed to about 10 per cent of the body weight of the host, animals were used for the experiment. Mice of third group were fed for a month on rice diet containing p-dimethylamino-azobenzene (DAB) at a level of 0.06 per cent. As controls, the mice of fourth group maintained on the same normal rice diet as the first and second groups. In all groups described above, water was freely and green vegetables were occasionally supplied.

All mice were sacrificed by decapitation and livers were removed promptly, and then chilled in ice water bath. To avoid any loss of activity, the assay of the TPO activity was started within 30 minutes after the death of the animals. The liver was homogenized with 9 volumes of cold 0.1M KCl solution, adjusted the acidity to pH 7.0 with 0.1N NaOH, by the aid of glass homogenizer. Usually 0.5ml of the homogenate was used in an enzyme assay. Assay of TPO activity was carried out by the similar method of Knox (2), and formed kynurenine was determined by the method of Mason and Berg (19).

For adaptation, 20mg of dl-tryptophan or 9mg of l-histidine per 10 g of body weight of mouse was injected intraperitoneally as a suspension or solution of 0.5 ml distilled water.

The catalase activity in the liver of tumor-bearing and control mice was determined by the aid of Battelli-Stern apparatus as described in previous papers (20, 21).

The results of levels of TPO in the liver of tumor-bearing, DAB-fed and control mice was summarized in Fig. 1. It may be clearly seen from Fig. 1, that the activity of TPO in the liver of tumor-bearing animals are significantly higher than

Range*	No. of Experiments	
.114–.252	7	Control
.178–.388	5	Tumor-bearing (S)
.190–.503	9	Tumor-bearing (I)
.119–.295	5	DAB-fed

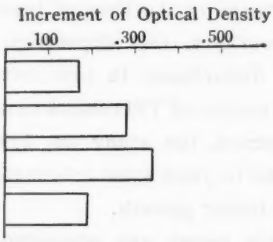

Fig. 1. TPO activity of livers of control, tumor-bearing and DAB-fed mice. Mice received the inoculation of Ehrlich ascites carcinoma subcutaneously (S) and intraperitoneally (I), respectively.
* Figures represent ranges of activity expressed as increment of optical density.

Range*	No. of Experiments	
5.4–9.9	7	Control
2.6–5.2	4	Tumor-bearing (S)
2.3–5.3	5	Tumor-dearing (I)
4.3–9.1	5	DAB-fed

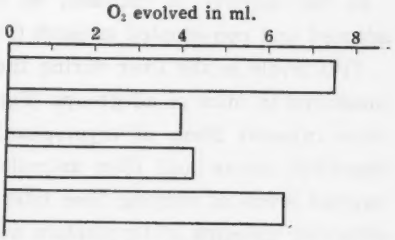

Fig. 2. Catalase activity of livers of control, tumor-bearing and DAB-fed mice.
* Figures represent ranges of activity expressed as ml of O_2 evolved.

that of control animals. While, the activity was around the normal level in DAB-fed animals. And the liver catalase of tumor-bearing animals decreased to the half level of control animals in its activity (Fig. 2). Since the TPO is required peroxide in its activity and consequently is inhibited by the high activity of catalase (12), so it is probable that the increased activity of TPO is resulted by the reduction of catalase activity in tumor-bearing animals. The diluted catalase solution prepared by the method of Sumner and Dounce (22) was added in the assay system of TPO of tumor-bearing animals to the extent of maximum activity of the liver catalase of control animals. But there was no significant change in the activity of TPO observed. From this fact, the increased activity of TPO in tumor-bearing animals may be independent on the activity of catalase in livers.

Range	No. of Experiments	
.641–.959	6	Control
.352–.662	6	Tumor-bearing (S)
.451–.772	5	Tumor-bearing (I)
.736–1.040	5	DAB-fed

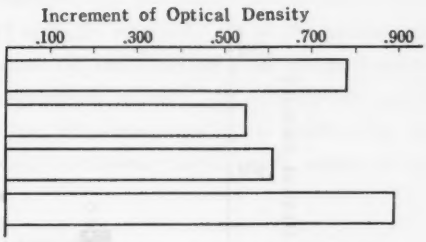

Fig. 3. TPO activity of livers of control, tumor-bearing and DAB-fed mice at 5 hours after the injection of tryptophan.

Fig. 3. shows the activity of TPO in the liver at 5 hours after the animal received the injection of tryptophan. Both activities of the TPO in control and DAB-fed mice were increased about 4 to 5 folds of the non-adapted, while the activity in tumor-bearing animals was increased only 2 folds of non-adapted animals. No significant differences were found in both the activity and the ability to induce the increase of the enzyme between in the liver of Tumor-bearing (I) and (S). The tumor cells were inert completely for kynurenine formation even after the tryptophan injected.

In the activity of catalase, no difference was observed between the livers of adapted and non-adapted animals (Fig. 4).

TPO levels in the liver during the period of substrate-induced adaptation were measured in mice of all groups (Figs. 5 and 6). In this experiment, the animals were injected 20mg of tryptophan per 10g of body weight intraperitoneally as described above and then animals were sacrificed for the determination of the enzyme levels at varying time intervals after the injection. Although the considerable amounts of tryptophan were remaining unabsorbed over the surface of the liver and in the peritoneal cavity at early periods, the remarkable increase in the activity was found at first 1 hour. Especially, in tumor-bearing animals, it was found that the enzyme level reached to a maximum in the liver within 1 hour

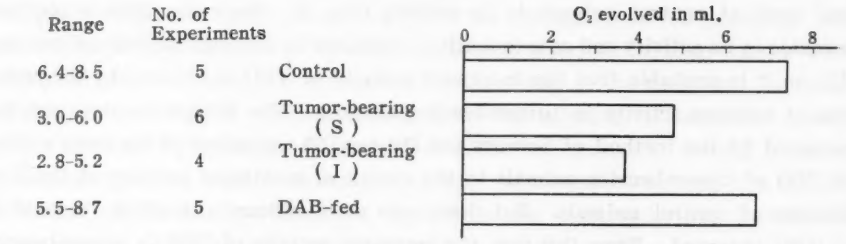

Fig. 4. Catalase activity of livers of control, tumor-bearing and DAB-fed mice at 5 hours after the injection of tryptophan.

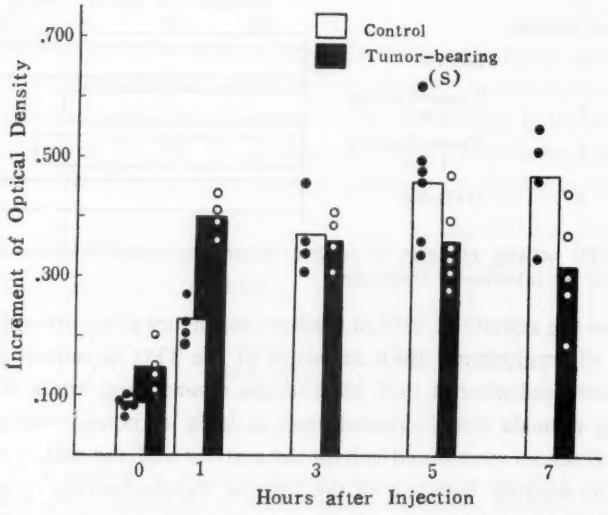

Fig. 5. TPO activity of liver of control and tumor-bearing mice during the tryptophan-induced adaptation.

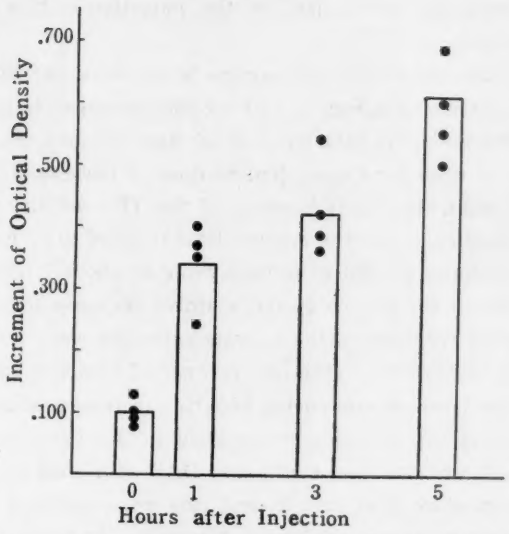

Fig. 6. TPO activity of liver of DAB-fed mice during the tryptophan-induced adaptation.

after the injection and then the activity remained its level for the following 2 hours and then decreased very gradually. On the other hand, the level of the enzyme in the liver of control animals reached the maximum at about 5 hours and at 1 hour after the injection the TPO activity was only 1/3 of its maximum.

In the liver of DAB-fed animals, the level of the enzyme rose in the similar tendency of control animals during the adaptation, namely, the activity increased gradually after the injection and reached the maximum level at 5 hours (Fig. 6).

The rapid adaptation of TPO in the liver of tumor-bearing animals seems to be

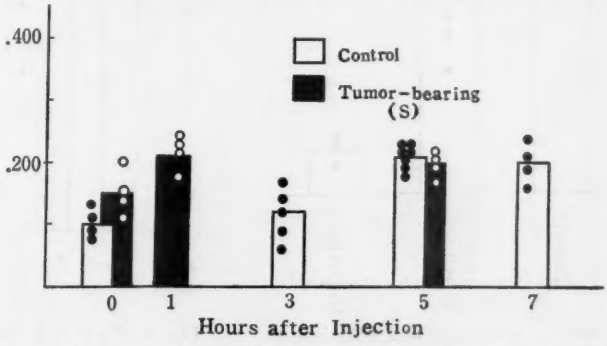

Fig. 7. TPO activity of liver of control and tumor-bearing mice during histidine-induced adaptation.

noteworthy, although the mechanism of the induction of this rapid adaptation still remains obscure.

As the hormone-induced adaptation, enzyme levels were measured by the injection of histidine. As shown in Fig. 7, the very poor response to histidine injection was observed in the mice. In rats, Knox *et al.* had reported the higher response of TPO than that of mice for the equivalent dose of histidine (3,23). In this experiment, a slight but a significant increase of the TPO activity was observed by the injection of histidine. And the enzyme level reached to its maximum at about 5 hours after the injection in control animals while at about 1 hour in tumor-bearing animals. Although the degree of the adaptive response to histidine injection was less than that of tryptophan, the enzyme activities were changed during the adaptation induced by histidine in the like manner of that induced by tryptophan.

2. Studies on the liver of rats during hepatic carcinogenesis. Male adult rats of Wister strain, weighing 120–150g, were used in this experiment. A group of rats was maintained on rice diet containing DAB at a level of 0.06 per cent for 4 months so as to produce liver cancer, and rats were returned to a normal rice diet without carcinogen for an additional 1 month. As a control group, Wistar

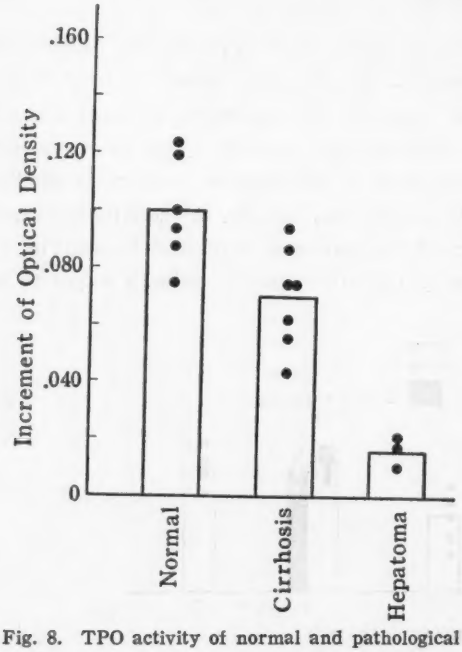

Fig. 8. TPO activity of normal and pathological rat liver.

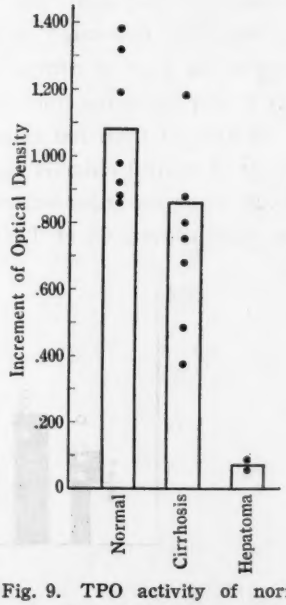

Fig. 9. TPO activity of normal and pathological rat liver at 5 hours after the injection of tryptophan.

rats were fed on the same diet without the carcinogen. In the both groups, water was freely and green vegetables were occasionally supplied.

Animals were exanguinated by cardiac puncture and livers were removed as quickly as possible, and placed on ice water bath and inspected. The livers were classified into two grades pathologically, cirrhosis and liver cancer, respectively. If the liver contained both cancerous areas and cirrhotic tissues, the different portions were dissected and handled separately.

Assay of the TPO activity was carried out in the same manner described above, except 1.0ml of the homogenate was used in the assay system. For adaptation, 200mg of dl-tryptophan or 90mg of l-histidine per 100g of body weight of rat was injected intraperitoneally.

The activities of TPO of the normal and pathological livers of non-adapted rats were summarized in Fig. 8. It may be clearly seen in the figure that the activity was decreased to 2/3 of normal when the liver shows cirrhotic. Moreover, when the tissue turned into cancerous, the activity was only in trace.

The activities of TPO of the livers of all rats at 5 hours after the tryptophan injection were increased markedly (Fig. 9). In the cirrhotic liver, the enzyme activity rose to 10 folds of the non-adapted as well as normal livers. From the result, the function of the synthesis of the enzyme in the liver was not disordered by the proceeding of the pathological changes during hepatic carcinogenesis. Although only a trace in the level of TPO activity was observed in the cancerous tissue, the activity was increased about 3 folds by the injection of the substrate.

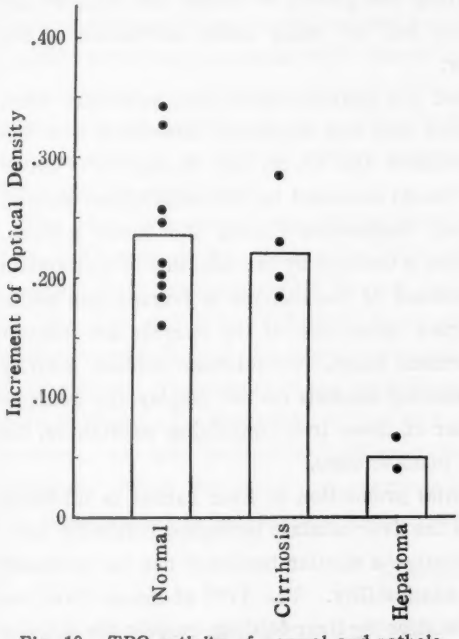

Fig. 10. TPO activity of normal and pathological rat liver at 5 hours after the injection of histidine.

Fig. 10. shows the result of the TPO sctivity after the histidine-induced adaptation. The injection of histidine induced the high level of TPO of the liver of all rats in the similar pattern as induced by the tryptophan injection, but the degree of the adaptive increment by histidine was less than that of increment induced by tryptophan.

Also in this case, the adaptive increment of the TPO activity was displayed in the cancerous tissues.

Discussion

The depression of the activities of the ferric enzymes such as catalase and cytochromes in the liver of tumor-bearing animals has been demonstrated by the previous investigators (24—27). From the results of this experiment, it is clear that the TPO in the mouse liver is not depressed but increased significantly by the inoculation of Ehrlich ascites carcinoma while the catalase activity decreased markedly in the liver of the same animals. Recently, Wood *et al.* reported that the TPO of mouse liver displays the biphasic changes during the growth of the inoculated carcinoma; at early period of tumor growth, the TPO decreased temporarily in its activity and then the TPO levels rose higher than those of the controls (36). And they suggested that the elevation of TPO activity was attributed to the result of the enhanced adrenocortical secretion during the tumor growth. But in this experiment, the histidine-induced adaptation in tumor-bearing mice was accomplished in the similar manner to that in control animals. Therefore, it is probable that the increased activity during the growth of tumor was induced not only by the increased adrenal function but by many other alterations of the metabolic process exerted by the tumor.

Nakahara and his associates separated the characteristic toxic substance from cancer tissue, toxohormone, and suggested that this substance interferes with the utilization of iron in the catalase synthesis (16, 17, 28, 29). In the TPO which contains iron in its configuration, the results obtained in this experiment showed that the activity remained without any depression during the tumor growth. However, when the new enzyme formation is induced by the injection of tryptophan within a relatively short period, the amount of the enzyme is formed less under the growth of the tumor. These complex behaviors of the enzyme are difficult to explain by the action of the toxohormone alone. Cytochrome oxidase activity and ferritin content of liver of tumor-bearing animals do not display the decreasing tendency (30, 31, 37). In the manner of these iron containing substances, the TPO seems to be out of the effects of toxohormone.

It is well known that the experimental production of liver cancer is inhibited by the liver feeding and in these cases the liver catalase increases markedly (38—40). A question may be raised as to whether a similar tendency can be produced by liver feeding on TPO level and its adaptability. The TPO of mouse liver exhibited the same tendency just as in catalase by liver feeding, namely the activity was increased about 2 folds of control but the degree of adaptation is not changed (Fig. 11).

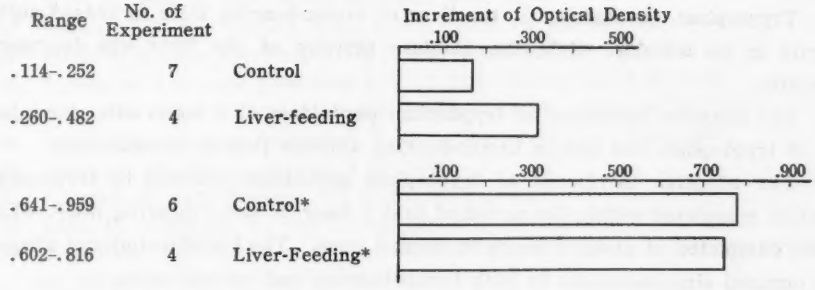

Fig. 11. TPO activity of livers of control and liver-fed mice.
Mice maintained on rice diet containing 5 per cent of dried powder of beef liver for a month used as Liver-feeding.
* TPO activity at 5 hours after the injection of tryptophan.

The fact that the duration of the adaptation is shortened by the growth of tumor—in tumor-bearing animals, namely, the activity of TPO reached the maximum within 1 hour after the injection and on the other hand, in control animals, it required at least 5 hours to complete the adaptation—seemed to be induced by the alteration of the protein metabolism in the liver of tumor-bearing animals. This alterations of the normal metabolism of protein in the liver of tumor-bearing animals have been demonstrated clearly by Norberg and Greenberg (14).

Kishi and his associates reported that some oxidases in the liver of rat were changed in its activity by the feeding of DAB for about 1 month (32-35). But in this experiment, no effect of feeding of hepatic carcinogen was found in both TPO level and its adaptability.

The changes in the activity of liver enzymes of rats fed DAB were demonstrated by many investigators. Especially, TPO of the liver of rats decreased in its activity during hepatic carcinogenesis in the manner of catalase and some oxidases (33, 34, 21). The significant reduction in the activity of TPO was seen when the liver showed cirrhosis, and the drastic decrease in the activity was recognized in the liver cancer. The extent of the adaptation of cirrhotic livers was retained at the normal range. Furthermore, in the liver cancer which showed only trace in TPO activity, the new enzyme formed considerably by the injection of tryptophan or histidine. From the result, it seems probable that the adaptability of the TPO for the stimulus is independent from its original activity in the liver.

Summary

1. Tryptophan peroxidase level and its activity after the injection of tryptophan or histidine were examined in the liver of tumor-bearing mice and hepatic carcinogen fed rats.

2. Tryptophan peroxidase in the liver of tumor-bearing mice increased significantly in its activity, while the catalase activity of the liver was depressed markedly.

3. The adaptive increment of tryptophan peroxidase at 5 hours after the injection of tryptophan was less in tumor-bearing animals than in normal ones.

4. The adaptive increment of tryptophan peroxidase induced by tryptophan injection completed within the period of first 1 hour in tumor-bearing mice, while it was completed at about 5 hours in normal ones. The histidine-induced adaptation occured simultaneously in both tumor-bearing and normal mice.

5. No remarkable changes were found in both the activity of tryptophan peroxidase and the ability to induce the increase of the enzyme between the mice fed hepatic carcinogen and the normal ones.

6. The reduction in the activity of tryptophan peroxidase was significant when the liver was cirrhotic. While the activity was negligible in the liver cancer.

7. The activity of tryptophan peroxidase during the adaptation in the cirrhotic liver retained at the normal degree.

8. The activity of tryptophan peroxidase in liver cancer increased significantly after the injection of tryptophan or histidine.

The auther is indebted to Professor Kazuo Mori for continuous aid and encouragement.

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要　旨

担癌動物の肝トリプトファンーペルオキシダーゼについて

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Ehrlich 腹水癌を移植した dd 系マウスについて肝のトリプトファンペルオキシダーゼーオキシダーゼの活性度ならびにその適応的活性の増加を測定した。同時に白鼠の DAB 肝癌生成過程における肝組織の同酵素の変化をも追求した。

担癌マウスの活性は対称動物に比し明らかに増加の傾向が見られたが同時に測定したカタラーゼの活性度は著減していた。

一方基質であるトリプトファンの注射による適応後の活性は担癌動物では正常のそれよりも低い値を示した。また基質注射後の適応的活性の増加を時間的に見ると担癌動物では注射後約1時間で最高値を示したが対称の正常動物では約5時間後に最高値に達した。すなわち担癌動物では適応が正常より明らかに短時間内に起っていた。この担癌動物における適応時間の短縮はヒスチヂン注射の場合にも見られた。

この酵素活性の適応的増加は新酵素蛋白の合成によることが証明されているのでこの結果は癌の成長にともない肝酵素蛋白の動きが早くなっていることを暗示しているように思われる。

一方 DAB による白鼠肝癌生成実験の過程における病変肝の酵素活性は硬変肝では正常肝の 2/3 であり肝癌では 1/6 を示すに過ぎなかった。しかし硬変肝でも基質およびヒスチヂンに対する適応力は正常肝と同程度に持っていた。さらに肝癌でも基質あるいはヒスチヂンの注入によってその活性が数倍の増加を示した。

(文部省科学研究費による)

[GANN, 49, 137~149 ; June, 1958]

TESTS OF ALLYL-SUBSTITUTED COMPOUNDS AGAINST VARIOUS MOUSE TUMORS*

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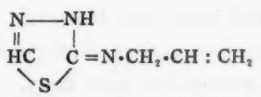
In 1889 Eykman (1) reported that chavicol (4-oxy-1-allyl-benzene) had a bacteriocidal activity five times stronger than phenol and twice that of eugenol (4-oxy-3-methyl-1-allyl benzene) on *Micrococcus B. B.*, *Staphylococcus aureus*, and other organisms.

A review of the literature on allyl-substituted compounds revealed the following reports. In 1953 Boyland and Sargent (2) noted that oral administration of allyl isothiocyanate mixed with arachis oil had no effect on Sarcoma MCDBl. Gellhorn, *et al.* (3) found that intraperitoneal injection of 1-allyl-theobromine had no inhibitory effect on mammary adenocarcinoma 755. Goodson, *et. al.* (4, 5) reported a negative effect of N-allyl-2-ethyl-2-hexene-1-imide and several related compounds on Sarcoma 180 and Adenocarcinoma E 0771. Stock, *et al.* (6, 7) also reported a negative effect of allyl-p-methoxy-benzoyl acrylate and several other related compounds on Sarcoma 180. Leiter, *et al.* (8) tested 1-allyl-4-(1, 2-phenylethyl)-piperazine·2HCl and related compounds on Sarcoma 37 and found them ineffective. Skipper and Thomson (9) observed that triallyl cyanurate had no effect on Adenocarcinoma 755.

Recently Endo synthesized a series of compounds, having in common the allyl group :—$CH_2 \cdot CH : CH_2$

A typical compound is



2-Allylimino-1, 3, 4-thiadiazoline

Endo and Furuta (10) tested *in vitro* the tuberculostatic activities of these compounds. Later Endo and Okamura (11) tested the inhibitory action of 14 of the water-soluble compounds on Ehrlich carcinoma and found that repeated intraperitoneal injection of 100mg/kg/day of 2-allylimino-1, 3, 4-thiadiazoline hydro-

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chloride caused a definite inhibitory effect on Ehrlich carcinoma (growth from 1/4 to 1/3 the average weight of the controls).

This report presents the anti-tumor studies with a large number of allyl-substituted compounds using a spectrum of tumors.

Materials and Methods

The tumors used in the present study are as follows: Sarcoma 180 (S 180), Bashford carcinoma 63 (C 63), Ehrlich carcinoma (EC), Harding-Passey melanoma (MM), in Rockland Swiss albino mice; Adenocarcinoma E 0771 (E 0771), Lewis bladder carcinoma (BC) and Lewis lung carcinoma (LC), Glioma 26 (G 26) in C 57 black mice; Sarcoma MA 387 (MA 387), Miyono adenocarcinoma (MC), Carcinoma 1025 (C 1025), Wagner osteogenic sarcoma (WOS), Ridgway osteogenic sarcoma (ROS), and Mecca lymphosarcoma (MLS) in AKR mice; and Gardner lymphosarcoma (GLS) in C3H mice. The history, biological properties, and cytological properties, and cytological descriptions of these tumors are given else-where (12, 13, 14).

Subcutaneous implantations of tumors (small pieces of tumor, each weighing approximately 6 mg) into healthy young animals (18- to 22- gm mice) were carried out by the usual trocar method with a single implant into the right axillary region. The progress of the tumors in the treated and untreated animals was recorded graphically by measuring the diameters of the tumors with calipers at weekly intervals for 4 weeks after tumor transplantation. The animals were maintained on a standard pellet diet (Purina Laboratory Chow) and water *ad ribitum.*

The first intraperitoneal injections of the compounds at or near maximum tolerated doses were given 24 hours after transplantation of the tumor and treatment was continued once daily for 7 days.

The compounds were dissolved in saline. However, if a compound was insoluble, it was homogenized with 0.5 per cent carboxymethylcellulose in saline. Usual injection volumes of the diluents were 0.5 cc once daily.

The degree of inhibition of the growth of the solid tumors was graded according to the following scheme:

− No effect; tumors in the treated animals grew on the average to 3/4 or more of the diameters of the control tumors.

± Slight inhibition; tumors in the treated animals grew to between 1/2 and 3/4 of the diameters of the controls.

+ Moderate inhibition; tumors in the treated animals grew to between 1/4 and 1/2 of the diameters of the controls.

++ Marked inhibition; tumors failed to grow or grew to 1/4 of the diameter or the controls.

Table 1. **Effect of Allyl-Substituted Compounds on Various 1-day-old Mouse Tumors**

SK No.	Compound	Tumor	Does mg/kg/day	No. of deaths		Av. wt. change (gm) treated/controls	Results of treatment	
				1st wk.	2nd wk.	1st week	1st wk.	2nd wk.
14, 556	Allylphenyl sulfide	S 180	125	0/5	0/5	+0.4/+0.5	−	−
		C 63	125	0/5	1/5	−1.5/−1.1	±	−
14, 557	2-Allylthiophenol	S 180	125	0/5	0/5	+0.3/+0.5	±	−
		C 63	125	0/5	0/5	0/−1.1	±	−
14, 558	Allylguaiacyl ether	S 180	125	0/5	0/5	+0.2/+0.5	±	−
		C 63	125	0/5	2/5	−3.2/−1.1	+	+
14, 559	*p*-Acetamidophenyl allyl ether	S 180	125	0/5	0/5	+0.4/+0.5	−	−
		C 63	125	0/5	1/5	−1.5/−1.1	±	+
14, 560	*p*-Aminophenyl allyl ether	S 180	125	1/5	1/5	−0.1/+0.5	−	−
		C 63	125	0/5	0/5	−0.3/−1.1	−	−
14, 561	*p*-Acetamido-*o*-allyl phenol	S 180	125	0/5	0/5	+0.1/ 0	±	±
14, 563	*m*-Acetamidophenyl allyl ether	S 180	125	0/5	1/5	+0.2, 0	−	−
		B C	125	0/5	0/5	−0.1/−0.1	−	−
14, 564	*o*-Allyl-5-acetamido-phenol	S 180	125	1/5	1/5	−1.0/ 0	±	−
14, 565	*m*-Aminophenyl allyl ether, HCl	S 180	125	0/5	0/5	+0.9/+1.4	−	−
		B C	125	0/5	0/5	+0.3/+1.1	−	−
14, 566	*o*-Allyl-5-amino-phenol, HCl	S 180	125	0/5	0/5	−0.4/+1.4	−	−
		B C	125	0/5	0/5	−0.5/+1.1	−	−
14, 567	*o*-Allyl-p-amino-phenol, HCl	S 180	125	0/5	0/5	+1.0/+1.4	−	−
		C1025	125	0/5	0/5	+0.8/+2.8	−	−
14, 568	*o*-Acetamidophenyl ether	S 180	125	0/5	0/5	+1.4/+1.4	−	−
		C1025	125	0/5	0/5	+0.1/+2.8	−	−
14, 569	*o*-Acetamido-6-allyl phenol	S 180	125	0/5	0/5	+1.1/+1.4	−	−
		C1025	125	0/5	0/5	−0.1/+2.8	±	±
14, 570	*o*-Aminophenol-6-allyl, HCl	S 180	125	0/5	0/5	+0.3/+1.4	−	−
		C1025	125	0/5	0/5	−0.4/+2.8	±	+
14, 571	*p*-Tolyl allyl ether	S 180	125	0/5	0/5	+2.1/+1.4	−	−
		M L S	125	0/5	0/5	+0.2/+1.3	−	−
14, 572	*o*-Tolyl allyl ether	S 180	125	0/5	0/5	−2.0/+0.5	−	−
		C 63	125	0/5	1/5	−0.9/+0.6	±	−
14, 573	Allyl (2-isoprophl-5-methyl)phenyl ether	S 180	125	0/5	0/5	−0.7/+0.5	−	−
		C 63	125	0/5	0/5	+0.2/+0.6	±	−

SK No.	Compound	Tumor	Does mg/kg/day	No. of deaths		Av. wt. change (gm) treated/controls	Results of treatment	
				1st wk.	2nd wk.	1st week	1st wk.	2nd wk.
14, 574	2-Allyl-6-isopropyl-*m*-cresol	C 63	125	4/5		−1.4/+0.6	too toxic*	
		MLS	125	3/5	4/5	−4.0/+2.2	too toxic	
		MLS	50	0/5	0/5	−0.8/+1.3	−	−
14, 575	*m*-Methoxyphenyl allyl ether	S 180	125	1/5	2/5	−1.5/+0.5	±	−
		MLS	125	2/5	2/5	−2.8/+2.2	−	−
14, 576	*o*-Allyl-5-methoxy-phenol	S 180	125	1/5	1/5	−1.8/+0.8	−	−
		C 63	125	1/5	1/5	−1.0/+0.6	±	±
		MLS	125	0/5	3/5	−2.7/+1.3	+	±
		BC	125	0/5	0/5	−1.4/−0.1	−	±
14, 577	*p*-Methoxyphenyl allyl ether	S 180	125	0/5	0/5	−2.4/+0.8	±	±
		C 63	125	1/5	1/5	−1.5/+0.6	±	±
		MLS	125	0/5	2/5	−2.6/+1.3	±	±
14, 578	*m*-Tolyl allyl ether	S 180	125	0/5	0/5	+0.4/+0.3	−	−
14, 579	6-Allyl-*m*-cresol	S 180	50	1/10	1/10	−0.3/+1.1	−	−
		C 63	50	1/5	1/5	−0.9/+0.6	±	±
14, 580	*m*-Hydroxyphenyl allyl ether	S 180	125	0/5	0/5	−0.6/+0.2	±	−
		MLS	125	0/5	1/5	−3.6/+1.8	±	±
14, 581	3-Allylcatechol, 1-monosodium salt	S 180	125	0/5	0/5	−0.7/+0.2	−	−
		MLS	125	0/5	0/5	+1.4/+1.8	−	−
14, 582	*o*-Allyloxybenzoic acid methyl ester	S 180	150	0/5	0/5	−0.3/+0.2	−	−
14, 583	*o*-Allyloxybenzoic acid, sodium salt	S 180	250	0/5	0/5	−0.4/+1.4	±	−
		S 180	125	0/5	0/5	+1.0/+1.5	−	−
		C 63	250	0/5	0/5	+0.5/+2.5	−	−
		C 63	125	0/5	0/5	+1.1/+1.2	−	−
14, 584	*o*-Allyloxybenzoic acid hydrazide	S 180	50	4/5	4/5	+0.2/+0.2	too toxic	
		S 180	25	1/5	1/5	−1.5/+1.4	−	−
		C 63	25	1/5	1/5	+0.5/+2.5	±	−
		WOS	25	1/5	1/5	+0.5/+2.0	−	−
14, 585	*o*-Allyloxy-*p*-amino-benzoic acid methyl ester, HCl	S 180	125	0/5	0/5	0/+1.5	−	−
		C 63	125	0/5	0/5	+0.2/+1.2	±	±
14, 586	*o*-Allyloxy-*p*-amino-benzoic acid	S 180	125	0/5	0/5	−2.6/+1.5	±	−
		C 63	125	0/5	0/5	−0.4/+1.2	±	−

SK No.	Compound	Tumor	Dose mg/kg/day	No. of peaths		Av. wt. change (gm) treated/controls	Results of treatment	
				1st wk.	2nd wk.	1st weet	1st wk.	2nd wk.
14, 587	1-Allyl-1-phenyl hydrazine, HCl	S 180	250	1/5	1/5	−1.2/+1.4	±	−
		S 180	125	0/5	0/5	+0.9/+1.5	−	−
		C 63	250	1/5	2/5	−1.2/+2.5	±	−
		C 63	125	0/5	0/5	+0.3/+1.2	−	−
14, 588	1-Allyl-1-(*o*-methoxyphenyl) hydrazine, HCl	S 180	125	0/5	0/5	+0.1/+1.5	±	−
		C 63	125	0/5	0/5	+0.6/+1.2	−	−
14, 589	*o*-Allylaminoanisole	S 180	125	0/5	0/5	−1.7/−2.5	±	−
		C 63	125	0/5	0/5	−0.3/+1.4	−	−
14, 590	Allylbenzene	S 180	125	0/5	1/5	+0.3/+0.5	±	−
		C 63	125	0/5	0/5	+0.2/−1.1	±	−
14, 591	*p*-Allylphenyl hydrazine	S 180	125	0/5	0/5	+0.3/+1.5	−	−
		C 63	125	0/5	0/5	+0.6/+1.2	−	−
14, 592	*p*-Allylaminoanisole	S 180	125	0/5	2/5	−0.2/+1.5	−	−
		C 63	125	0/5	1/5	+0.3/+1.2	−	−
14, 593	N-Allyl-*o*-toluidine	S 180	125	0/5	1/5	+0.3/+1.4	−	−
		S 180	125	0/5	2/5	+1.8/+2.3	−	−
		C 63	250	0/5	1/5	−0.3/+2.5	±	±
14, 594	N-Allyl-*p*-toluidine	S 180	125	0/5	1/5	−4.0/+2.3	±	−
		R O S	125	0/5	0/5	−5.1/+0.7	±	−
14, 595	N-Allylaniline	S 180	200	0/5	2/5	+0.5/+1.0	−	−
		C 63	200	0/5	0/5	−1.0/+1.0	+	±
		C L S	200	0/5	2/5	−1.2/−0.1	−	±
14, 596	5-Allylaminophenol	S 180	125	1/5	0/5	−0.2/+2.3	−	−
		C 63	125	0/5	2/5	+1.1/+2.7	−	−
14, 597	N-Allyl-*m*-toluidine	S 180	250	0/5	1/5	+0.9/+1.4	±	−
		S 180	125	0/5	0/5	+1.4/+2.3	−	−
		C 63	250	0/5	0/5	+0.2/+2.5	±	±
		C 63	125	0/5	0/5	+0.9/+2.7	−	−
14, 598	*p*-Allylaminophenol	S 180	125	0/5	1/5	−0.9/+2.3	−	−
		C 63	125	0/5	0/5	+0.5/+2.7	−	±
14, 599	6-Allylaminophenol	S 180	250	0/5	1/5	−1.6/+1.4	±	−
		S 180	125	0/5	2/5	+1.3/+2.3	−	−
		C 63	250	0/5	1/5	−1.7/+2.5	±	−
		C 63	125	0/5	0/5	+1.9/+2.7	−	−
14, 600	N-Acetyl-N'-allyl-*p*-phenylenediamine	S 180	250	0/5	0/5	+2.1/+1.4	±	−
		S 180	125	0/5	2/5	−0.1/+2.3	−	−
		C 63	250	1/5	1/5	+1.2/+2.5	±	±
		C 63	125	0/5	0/5	+2.2/+2.7	−	−

SK No.	Compound	Tumor	Dose mg/kg/day	No. of deaths		Av. wt. change (gm) treated/controls	Results of treatment	
				1st wk.	2nd wk.	1st week	1st wk.	2nd wk.
		MLS	125	0/5	2/5	−0.9/+3.9	−	−
		CLS	125	0/5	1/5	−2.2/−0.3	−	−
14, 601	N-Allyl-*p*-phenylene-diamine	S 180	50	0/5	1/5	+0.5/+0.9	−	−
		C 63	50	0/5	0/5	−0.6/+1.4	±	−
		LC	50	1/5	1/5	+0.3/−0.1	−	−
		MLS	50	0/5	3/5	+0.8/+2.9	−	−
14, 602	N-Allyl-*p*-toluene sulfobamide	S 180	125	0/5	2/5	−2.2/−2.5	+	−
		C 63	125	0/5	0/5	−1.0/+1.4	±	−
14, 603	*p*-Allylaminoaceto-phenone	S 180	125	0/5	2/5	−2.2/−2.5	−	−
		C 63	125	0/5	0/5	0/+1.4	±	±
		LC	125	0/5	0/5	+0.1/−0.1	−	−
14, 604	N, N'-Diallyl-*p*-phenyl-enediamia-mine	S 180	50	0/5	3/5	−0.2/+1.0	−	−
		C 63	50	0/5	0/5	+0.1/+1.0	±	±
		GLS	50	0/5	0/5	−2.8/−0.1	±	±
14, 605	N, N'-Diallylbenzi-dine	S 180	125	0/5	0/5	−1.1/−2.5	±	−
		C 63	125	0/5	0/5	−1.2/+1.4	−	±
14, 606	N-Allylbenzidine	S 180	125	0/5	1/5	−0.9/−2.5	−	−
		C 63	125	1/5	1/5	−1.7/+1.4	±	−
14, 607	*p*-Allylaminoazo-benzene	S 180	125	0/5	1/5	−3.2/−2.5	+	−
		C 63	125	2/5	2/5	−3.3/+1.4	+	−
14, 608	N-Allyl-β-naphthyl-amine	S 180	300	2/5	2/5	−1.5/−0.4	−	−
		C 63	300	0/5	0/5	−2.9/+0.6	−	−
14, 609	N-Allyl-α-naphthyl amine	S 180	100	0/5	2/5	−0.7/+1.0	−	−
		C 63	100	0/5	0/5	+0.4/+1.0	−	−
		GLS	100	0/5	0/5	−2.1/−0.1	±	±
14, 610	*p*-Allylaminobenzoic acid hydrazide·HCl	S 180	125	0/5	1/5	−1.8/−1.6	±	−
		ROS	125	0/5	1/5	−1.0/+0.7	−	−
14, 611	*p*-Allylaminobenzoic acid	S 180	125	1/5	1/5	−0.2/−1.6	−	−
14, 612	*p*-Allylaminopyridine	S 180	10	0/5	0/5	+0.5/−0.4	±	−
		C 63	10	0/5	1/5	−0.2/+0.6	±	−
		G 26	10	1/5	1/5	−0.7/+0.1	∓	±
14, 613	4-Allylaminophenyl-acetonitrile	S 180	25	0/10	3/10	−0.3/+0.9	−	−
		C 63	25	0/5	0/5	0/0	−	±

SK No.	Compound	Tumor	Dose mg/kg/day	No. of deaths		Av. wt. change (gm) treated/controls 1st week	Results of treatment	
				1st wk.	2nd wk.		1st wk.	2nd wk.
		MA387	25	1/5	1/5	+1.0/+1.3	±	−
		E0771	25	0/5	0/5	−0.6/0	−	−
		MC	25	0/5	0/5	+0.8/+1.7	±	−
		C1025	25	0/5	0/5	−0.6/+3.3	−	±
		BC	25	0/5	1/5	−0.5/+0.7	−	−
		LC	25	0/5	0/5	+0.1/+0.5	−	−
		WOS	25	0/5	0/5	+1.3/+2.2	−	−
		ROS	25	0/5	0/5	+1.4/+1.3	−	+
		MLS	25	0/10	0/10	0.7/+1.6	±	±
		GLS	25	1/5	1/5	−2.1/−0.3	−	−
14, 614	N, N'-Diallyl-*o*-tolidine	S 180	50	1/10	3/10	−1.8/+0.6	±	−
		C 63	50	0/5	0/5	−1.6/+2.7	±	−
		LC	50	0/5	1/5	−2.0/+0.5	±	±
		ROS	50	0/5	0/5	−0.8/+0.7	±	+
14, 615	*p*-Allylaminobenzamide·HCl	S 180	100	0/10	4/10	−1.3/−0.8	±	−
		C 63	100	0/5	0/5	−0.4/+0.6	−	−
		G 26	100	0/5	0/5	−1.0/+0.1	±	−
14, 616	*p*-Allylaminophenyl allyl ether	S 180	125	0/5	0/5	−1.7/−1.6	−	−
		MLS	125	1/5	2/5	+0.6/+3.9	±	−
14, 618	*o*-Allylaminophenyl allyl ether	S 180	125	0/5	1/5	−1.8/−1.6	−	−
		C 63	125	1/10	3/10	−0.8/+2.9	−	−
		WOS	125	0/5	1/5	−2.9/+2.0	−	±
		ROS	125	0/5	0/5	−1.9/+0.7	±	±
14, 619	*p*-Allylamino benzene sulfonamide	S 180	125	0/5	2/5	−1.8/−2.5	±	±
		C 63	125	0/5	0/5	0/+1.4	−	−
		LC	125	0/5	0/5	−0.1/−0.1	−	−
14, 620	*p*-Allylaminobenzaldehyde thiosemicarbazone	S 180	125	0/5	2/5	+0.9/−1.6	−	−
14, 621	5-Allylamino-*o*-cresol	S 180	125	0/5	1/5	−1.8/+0.7	−	−
		C 63	125	0/5	0/5	+1.7/+1.6	±	±
14, 622	6-Chloro-N-allyl-aniline	S 180	250	0/5	1/5	−2.9/+0.3	−	−
		S 180	125	0/10	3/10	−1.5/+0.3	−	±
		MLS	250	1/5	2/5	−0.6/+2.4	−	±
		C 63	125	0/5	0/5	+1.2/+2.4	−	±
14, 623	*m*-Allylamino-*p*-cresol	S 180	125	0/5	1/5	−1.4/+0.7	−	−
		C 63	125	0/5	0/5	+1.5/+1.6	±	−

SK No.	Compound	Tumor	Does mg/kg/day	No. of deaths		Av. wt. change (gm) treated/controls 1st week	Results of treatment	
				1st wk.	2nd wk.		1st wk.	2nd wk.
		BC	125	0/5	0/5	−0.1/+0.7	−	−
14, 624	6-Allylamino-*m*-cresol	S 180	125	0/5	1/5	−1.3/+0.7	±	−
		C 63	125	0/5	0/5	−1.0/+1.6	−	±
14, 625	*p*-Allylamino-*m*-cresol	S 180	125	0/5	0/5	−0.8/+0.7	±	−
		C 63	125	0/5	0/5	+0.1/+1.6	±	±
14, 626	N-Allyl-*m*-nitro-*p*-toluidine	S 180	125	0/5	1/5	−1.4/+0.7	±	−
		C 63	125	0/5	1/5	+0.9/+1.6	±	−
14, 627	*p*-Allylamino-*o*-cresol	S 180	125	0/5	1/5	−1.5/+0.7	−	−
		C 63	125	1/5	2/5	−1.2/+1.6	±	−
14, 628	N-Allylthiourea	S 180	250	0/5	1/5	−1.0/+0.3	−	−
		S 180	125	0/5	1/5	−1.1/+1.7	−	−
		MLC	250	0/5	0/5	+1.5/+2.4	−	−
		C 63	125	0/5	0/5	+1.4/+2.4	−	−
14, 630	4-Allylthiosemicarbazide	S 180	5	1/5	1/5	+0.6/−0.4	+	±
		C 63	5	0/5	0/5	−0.2/+0.6	−	−
		E0771	5	0/5	0/5	−0.4/−0.9	±	−
14, 631	4-Allyl-1-formylthiosemicarbazide	S 180	200	0/5	0/5	+2.6/+1.8	±	±
		C 63	200	0/5	0/5	−1.0/+1.0	+	±
		LC	200	0/5	0/5	−0.2/+0.6	−	−
		MLS	200	0/5	3/5	+0.4/+0.8	−	−
14, 632	2-Allylimino-1, 3, 4-thiadiazoline, HCl	S 180	100	1/10	2/10	−2.6/+0.5	±	−
		C 63	100	0/10	2/10	−3.2/+1.6	+	±
		MA387	100	0/5	0/5	−2.9/+1.3	±	±
		EC	100	2/10	3/10	−2.7/+1.6	+	±
		E0771	100	0/5	0/5	−2.2/0	±	±
		MC	100	0/5	1/5	−3.7/+1.7	+	+
		C1025	100	0/10	0/10	−4.9/+3.0	+	+
		BC	100	0/5	0/5	−3.6/+0.2	±	±
		LC	100	3/10	3/10	−1.6/+0.4	+	±
		WOS	100	0/5	0/5	−4.7/+2.2	±	+
		ROS	100	0/5	0/5	−3.6/+1.3	−	+
		MLS	100	1/10	1/10	−1.8/+1.4	∓	+
		GLS	100	0/10	1/10	−3.2/−0.2	±	+
		MM	100	0/5	0/5	−1.8/+1.5	±	+
		G 26	100	2/5	2/5	−1.6/+0.1	+	±
14, 633	2-Allylimino 5-methyl-1, 3, 4-thiadiazoline	S 180	100	0/5	0/5	+0.3/+1.8	±	−
		C 63	200	0/5	0/5	−2.2/+1.0	±	−

SK No.	Compound	Tumor	Dose mg/kg/day	No. of deaths		Av. wt. change (gm) treated/controls 1st week	Results of treatment	
				1st wk.	2nd wk.		1st wk.	2nd wk.
		M L S	200	2/5	3/5	+0.4/+0.8	−	−
14, 634	6-Allylaminoquinoline	S 180	50	0/5	2/5	+0.7/+1.0	−	−
		E 0771	50	0/5	0/5	−0.7/0	−	±
		G L S	50	0/5	0/5	−0.8/−0.1	±	±
14, 635	N-Allyl-N'-(*p*-tolyl) thiourea	S 180	250	2/5	4/5	−1.1/+0.3	−	−
		S 180	125	0/5	0/5	−1.0/+1.7	−	±
		M L S	250	2/5	2/5	+0.3/+2.4	−	−
		C 63	125	0/5	0/5	+1.1/+2.4	±	−
14, 636	N-Allyl-N'-(*p*-methoxyphenyl) thiourea	S 180	250	2/5	2/5	+1.1/+0.3	−	−
		S 180	125	1/5	1/5	−0.7/+1.7	±	−
		M L S	250	2/5	2/5	+1.1/+2.4	−	−
		C 63	125	0/5	0/5	+1.0/+2.4	−	±
14, 637	N-Allyl-N'-(*o*-hydroxy-phenyl)thiourea	S 180	250	4/5	4/5	−2.4/+1.4	too toxic	
		S 180	125	0/10	3/10	+0.1/−0.2	−	−
		C 63	125	1/5	1/5	+2.3/+1.6	±	−
14, 638	N-Allyl-N'-*p*-sulfonamidophenyl) thiourea	S 180	125	0/5	1/5	−0.7/+0.7	−	−
		C 63	125	0/5	0/5	+1.3/+1.6	±	−
14, 639	1-(Allylcarbothiamido) piperidine	C 63	125	1/5	2/5	−0.2/−1.1	±	±
14, 640	2-Allylimino-1, 3, 4-thiadiazoline-Na-oxide	S 180	100	1/5	1/5	+0.4/−0.4	±	−
		C 63	100	1/5	1/5	−2.3/+0.6	±	−
		E C	100	0/5	0/5	−2.4/−2.9	+	±
		B C	100	0/5	0/5	+0.1/+0.2	±	−
		E 0771	100	0/5	0/5	0/−0.9	+	±
		M L S	100	0/5	3/5	+0.4/+0.3	±	−
14, 640	2-Allylimino-1, 3, 4-thia-diazoline-Na-oxide (concluded)	G L S	100	0/5	0/5	−0.5/+0.3	−	−
		G 26	100	0/5	0/5	+0.2/+0.1	+	−
14, 641	2-(γ-Chloroallylimino)-1, 2, 3-thiadiazoline	S 180	100	0/5	0/5	+0.7/+1.8	±	−
		C 63	100	0/5	0/5	0/+1.0	−	−
		L C	100	0/5	0/5	−0.3/+0.6	±	−
		M M	100	0/5	0/5	−2.5/+1.5	±	+
14, 642	4-(γ-Chloroallyl)thiosemicarbazide	S 180	125	0/5	0/5	+0.1/−0.7	±	±
		M L S	125	0/5	0/5	+2.2/+2.9	±	−

* Too toxic is indicated by death of more than 40 per cent animals.

In all tables the average weight change in grams of treated animals over control animals after one week of treatment is recorded. Five or 10 animals were used in each test group. The results presented in Table 1 are the averages of combined groups. All the results with Sarcoma 180 are evaluated only on the basis of first-week results.

Results

The results obtained with allyl-substituted compounds tested at or near maximum tolerated doses are summarized below and in Table 1.

The average percentage of tumor regressions in control animals were 0 (Ehrlich carcinoma) to 7 (Wagner osteogenic sarcoma).

It is apparent from the data that these allyl-substituted compounds are not strong anti-tumor agents. Among 85 compounds tested, 9 showed a definite inhibitory effect on the growth of one or more tumors. Thus repeated intraperitoneal injections of 100mg/kg/day of 2-allylimino-1, 3, 4-thiadiazoline·HCl (SK 14,632) had a moderate inhibitory effect on Miyono adenocarcinoma, Carcinoma 1025, Wagner osteogenic sarcoma, Ridgway osteogenic sarcoma, Mecca lymphosarcoma, Gardner lymphosarcoma, and Harding-Paseey melanoma, while it had a slight inhibitory effect on Sarcoma 180, Sarcoma MA 387, Ehrlich carcinoma, Bashford carcinoma 63, Lewis bladder carcinoma, Lewis lung carcinoma and Glioma 26.

Table 2. The Growth Inhibitory Activities of 6-Mercaptopurine and 2-Allylimino-1, 3, 4-Thiadiazoline·HCl on Various Mouse Tumors

Tumor	6-MP 30mg/kg/day	SK 14,632 100mg/kg/day
Sarcoma 180	###	−
Sarcoma MA 387	±	±
Ehrlich carcinoma	+	±
Bashford carcinoma 63	##	±
Adenocarcinoma E 0771	##	±
Miyono adenocarcinoma	+	+
Carcinoma 1025	##	+
Lewis bladder carcinoma	−	±
Lewis lung carcidoma	−	±
Wagner osteogenic sarcoma	+	+
Ridgway osteogenic sarcoma	##	+
Mecca lymphosarcoma	−	+
Gardner lymphosarcoma	−	+
Harding-Passey melanoma	−	+
Glioma 26	##	±

− in dicates no effect; + indicates moderate inhibition; ## indicates marked inhibition; ### indicates complete inhibition or destruction of tumor.

N-allyl-p-toluene sulfonamide (SK 14,602), *p*-allyl-aminoazobenzene (SK 14,607), and 4-allyl thiosemicarbazide (SK 14,630) had a moderate inhibitory effect on Sarcoma 180; allylguaiacyl ether (SK 14,558) on Bashford carcinoma 63; 6-allyl-*o*-aminophenol·HCl (SK 14,570) on Carcinoma 1025; 4-allylaminophenylacetonitrile (SK 14,613), and N, N'-diallyl-*o*-tolidine (SK 14,614) on Ridgway osteogenic sarcoma, and 2-(*γ*-chloroallylimino)-1, 3, 4-thiadiazoline (SK 14,641) on Harding-Passey melanoma. All other 76 compounds caused only slight or no inhibitory effects.

It is of interest to compare the anti-tumor action of 2-allylimino-1, 3, 4-thiadiazoline·HCl with a good anti-tumor compound. For this we selected 6-mercaptopurine because it had a destructive effect on Sarcoma 180 and significantly inhibited the growth of several other mouse tumors (15,16).

From the results summarized in Table 2, it is clear that 2-allyl-imino-1, 3, 4-thiadiazoline was less effective than 6-mercaptopurine although the former had a definite inhibitory effect on the growth of 7 mouse tumors.

Discussion

2-Allylimino-1, 3, 4-thiadiazoline hydrochloride is a water-soluble toxic substance. Its toxicity appears to be accumulative during the injection period. Sometimes, mice in which tumor growth was inhibited markedly died later of the accumulative toxicity.

In 1955 Oleson, *et al.* (17) found that 2-amino-1, 3, 4-thiadiazole was most effective on Melanoma S 91, Glioblastoma S 110, and Lymphosarcoma 6C3HED among 11 similar compounds tested. Burchenal and Dagg (18) reported that 2-ethylamino-1, 3, 4-thiadiazole (SK 14,406) prolonged the survival time of mice with Leukemia 8174 at doses of 50-100mg/kg injected intraperitoneally 3 times weekly. Its chemical constitution is very similar to that of 2-allylimino-1, 3, 4-thiadiazoline. N-allyl-*β*-naphthylamine (SK 14,608) inhibited the growth of tubercle bacilli in the dilution of 1/1,000,000 ***in vitro*** (10), but it had no inhibitory effect on the growth of Sarcoma 180 and Bashford carcinoma 63.

Summary

1. The effects of allyl-substituted compounds have been tested against a spectrum of 15 tumors of the mouse.

2. Among the series of 85 allyl-substituted compounds tested, 2-allylimino-1, 3, 4-thiadiazoline hydrochloride showed the most anti-tumor activity. At daily doses of 100mg/kg it had a moderate inhibitory effect on Miyono adenocarcinoma, Carcinoma 1025, Wagner and Ridgway osteogenic sarcomas, Gardner lymphosarcoma, and Harding-Passey melanoma. and a slight inhibitory effect on Sarcoma 180, Sarcoma MA 387, Ehrlich carcinoma, Bashford carcinoma 63, Adenocarcinoma E0771,

Lewis bladder carcinoma, Lewis lung carcinoma, and Glioma 26.

3. N-Allyl-*p*-toluene sulfonamide, *p*-allylaminoazobenzene, and 4-allylthiosemicarbazide had a moderate inhibitory effect on Sarcoma 180; allylguaiacyl ether had a moderate inhibitory effect on Bashford carcinoma 63; 6-allyl-*o*-aminophenol·HCl had a moderate inhibitory effect on Carcinoma 1025; 4-allylaminophenylacetonitrile and N, N'-diallyl-*o*-tolidine had a moderate inhibitory effect on Ridgway osteogenic sarcoma; and 2-(γ-chloroallylimino)-1, 3, 4-thiadiazoline had a moderate inhibitory effect on Harding-Passey melanoma.

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要　　旨

Mouse tumor に対する allyl 化合物の制癌作用

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Trocar 法により，allyl 化合物 85 種 (遠藤合成) の mouse tumor 15 種に対する制癌作用を試験した。約 6 mg の tumor 片をマウスの右腋下皮下に移植し，翌日より連続 7 日間，薬剤の生食溶液 (あるいは carboxymethyl cellulose の suspension 液) 0.5 cc を腹腔内に注射した。制癌成績は薬剤注射終了日およびさらに 1 週間後に caliper にて tumor size を測り観察した。

SK 14,632 2-allylimino-1, 3, 4-thiadiazoline HCl が制癌作用もっとも強く，tumor size が対照の 1/4 程度のものが 7；1/2 程度のものが 7 あった。

[GANN, 49, 151~156; June, 1958]

ON THE RELATION BETWEEN CARCINOGENIC POTENCY OF 4-NITROQUINOLINE N-OXIDES AND THE REACTIVITY OF THEIR NITRO-GROUPS WITH SH-COMPOUNDS

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In 1957, Nakahara, Fukuoka and Sugimura succeeded in producing skin cancer in mice by the application of 4-nitroquinoline N-oxide (1). This substance is of special interest not so much because its carcinogenicity is almost as strong as the most powerful known carcinogens, such as methylcholanthren, etc., but because it belongs structurally to hetero-bicyclic compounds, among which no carcinogen of power has hitherto been found. Further investigations by Nakahara and co-workers, using 4-nitroquinoline N-oxide and its derivatives, showed that the nitro-group in the position 4 and N-oxide group of the quinoline nucleus are indispensable for the carcinogenic action of the molecules (2).

As well known the polar nitro-group at the position 4 of this compound, through the strong polar effect of the N-oxide group, forms a highly nucleophilic reactive center., and Okabayashi (3) demonstrated that the 4-nitro-group of quinoline N-oxide can be easily substituted with SH-group, using thioglycolic acid as well as cystein under physiological conditions.

On the other hand, Fukuoka and co-workers established that the glycolysis of tumor cells is strongly inhibited by the addition of 4-nitroquinoline N-oxide, while respiration remained practically unaffected (4). From these facts it is possible to assume that the glycolytic inhibition comes about as a result of the substitution reaction between 4-nitroquinoline N-oxide and a functional group (SH-group) of glycolytic enzymes.

In this paper will be reported first our experimental data which indicate that there is a definite relation between the carcinogenicity of 4-nitroquinoline N-oxides and the reactivity of their 4-nitro-groups with SH-group. Our experimental results on the similar reaction of 4 nitroquinoline N-oxide with such other important cell components as amino acids and nucleic acids will also be described.

MATERIAL AND METHODS

The following NO_2 compounds were used in this study : 4-nitroquinoline N-oxide, 6-chloro-4-nitroquinoline N-oxide, 4-nitroquinaldine N-oxide, 4-nitro-2-ethylquino-

line N-oxide, 4-nitroquinoline, 6-nitroquinoline and 4-nitropyridineN-oxide.

All but the last compound have already been tested as to their carcinogenicity by Nakahara and his co-workers (2), who showed that the first four are strongly carcinogenic and the fifth and sixth compounds non-carcinogenic. The result of the carcinogenesis experiment with 4-nitropyridine N-oxide has not been published, but according to personal communication from Dr. Nakahara, it is non-carcinogenic.

The SH compound used were glutathione and cystein.

I. a) The determination of the reactivity of the nitro-group with SH group was carried out in the following manner:

The reaction mixture was set up as 1 follows:

10^{-2}M R-SH	4ml.
5×10^{-2}M (in absolute alcohol) Quinoline or pyridine	4ml.
10^{-2}M Ethylenediaminetetraacetate (EDTA)	1ml.
10^{-1}M Phosphate buffer (pH 7.0)	1ml.

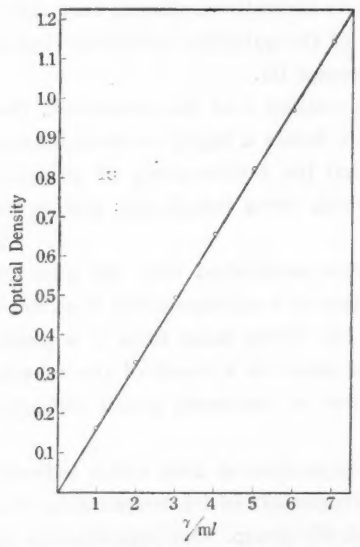


Figure 1. Standard Curve of $NaNO_2$ after Diazo Reaction. As solvent for $NaNO_2$ a mixture of 4 parts alcohol, 4 parts distillled water, 1 part EDTA and 1 part 10^{-1}M phosphate buffer (pH 7.0) was used. This solution with calculated concentration of $NaNO_2$ was mixed with equal amount of diazo reagent, kept at 0°C for one hour, and then measured spectrophotometrically at the wave-length of 480mμ.

The reaction mixture was kept at 37°C in water bath for 1 hour, after which 0.5ml. of it was diluted with 4ml. of 5×10^{-1}M tris buffer (pH 7.4), 0.2ml. EDTA and 3.3ml. distilled water, and was amperometrically titrated with 10^{-3} M $AgNO_3$. In this way the amount of the SH group remaining unconsumed after the reaction was determined (5).

b) At the same time, 3ml. of diazo reagent, (6) consisting of 0.1 percent sulphanilic acid, 0.08 percent α-naphtol and 75 percent acetic acid, was added to 3ml. of the above described reaction mixture and was kept at 0°C. After one hour the intensity of the color was measured spectrophotometrically at the wave-length of 480mμ, and the amount of the HNO_2 liberated was calculated by refer-

ring to the following standard curve (Figure 1).

II. For the observation of the reaction between 4-nitroquinoline N-oxide and some of the more important cell components, the following substances were used as experimental models:

L-Alanine, L-leucine ethylester, thymine, ATP, RNA and DNA.

Nine ml. of 10^{-4}M solution of the substance to be tested in 10^{-1}M phosphate buffer of pH 7.0 (0.1 percent solution in the case of RNA and DNA) was added to 1ml. of 5×10^{-4}M alcoholic solution of 4-nitroquinoline N-oxide, and the mixture was allowed to stand for one hour at 37°C. The reaction mixture was then examined spectrophotometrically to see if there was any change in the absorption curve of 4-nitroquinoline N-oxide in comparison with that of the blank control.

RESULTS

Table 1 shows the amount of 10^{-3}M $AgNO_3$ consumed by SH group which remained after the reaction for 1 hour in Experiment I a.

Here, as contol, 4ml. of distilled water or 10^{-3}M HNO_2 was used, in place of 5×10^{-3} solution of test substance.

Table 1. Amperometric Titration (Experiment I a)

	Cystein	Glutathione
Blank	0.235	0.190
10^{-3}M HNO_2	0.225	0.177
6-Chloro-4-nitroquinoline N-oxide	0.012	0.017
4-Nitroquinoline N-oxide	0.050	0.030
4-Nitroquinaldine N-oxide	0.150	0.132
4-Nitro-2-ethylquinoline N-oxide	0.173	0.150
4-Nitroquinoline	0.180	0.160
4-Nitropyridine N-oxide	0.207	0.170
6-Nitroquinoline	0.223	0.175

The difference in the amount of consumed $AgNO_3$ in the two controls is very small, which shows that the concentration of the split HNO_2 produced under our experimental conditions offers no great obstacle, in spite of the conceivable oxidation of SH-group by HNO_2.

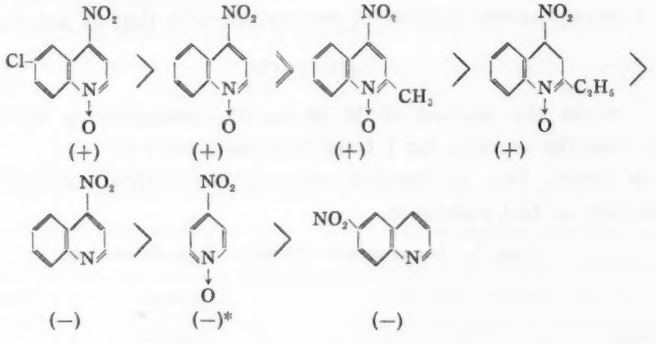
Table 2 brings together the amounts of the split HNO_2, calculated from the standard curve. (Experiment I b). These represent the amounts of substitution of nitro-group with SH-group. The case of 4-nitroquinoline is not included in this table, since it was impossible to make colorimetric titration in this case on account of the diazo reaction already taking place in control mixtures.

From the results described above the quinoline and pyridine derivatives examined in this investigation may be listed as follows, according to the degree of their

Table 2. Colorimetric Determination (Experiment I b)

	Cystein	Glutathione
6-Chloro-4-nitroquinoline N-oxide	25.8γ	30.2γ
4-Nitroquinoline N-oxide	19.1γ	19.8γ
4-Nitroquinaldine N-oxide	7.1γ	5.5γ
4-Nitro-2-ethylquinoline N-oxide	6.0γ	5.9γ
4-Nitropyridine N-oxide	0.7γ	1.2γ
6-Nitroquinoline	0	0

reactivities with SH group:



(+) and (−) in the above list indicate carcinogenic and non-carcinogenic, respectively, according to Nakahara and co-workers (2). * indicates unpublished, personal information from Dr. Nakahara.

Table 3 represents the result of Experiment II, and shows the optical density and λmax which were given by the reaction mixtures (pH 7.0) after standing for one hour at 37°C.

Table 3. λmax and Optical Densities

	4-Nitroquinoline N-oxide alone (Control)	+ Alanine	+Leucine ethyl ester	+ Thymine	+ATP	+RNA	+DNA
λmax. mμ	370	370	370	370	370	370	370
Optical density	0.450	0.440	0.438	0.440	0.448	0.452	0.455

DISCUSSION

The first part of our experiments (Experiment I, a and b), demonstrated a very interesting fact that those quinoline derivatives known to have carcinogenic action show stronger reactivity of their nitro-group with SH-group. Although it is not possible to establish a complete parallelism between this reactivity and carcinogenic

potency, because of the qualitative nature of the available data from the carcinogenesis experiments, it can safely be stated that more than a certain degree of the reactivity is required for the carcinogenicity of this class of nitroquinoline compounds.

From their great facility for the substitution reaction, it can easily be assumed that these compounds readily react non-enzymatically with the SH-group of the cell, including that of the cell membrane.

A question arises as to whether a quinoline N-oxide derivative with more reactive nitro-group at the position 4 is more active carcinogenically than 4-nitroquinoline N-oxide, and whether other compound with just as strong a nucleophilic reactive center, such as 2, 4-dinitrobenzenesulfonic acid or 2, 4-dinitrofluorobenzene can also be carcinogenic. Further investigation is needed to answer these questions.

The results of the second part of this study (Experiment II) showed that amino acids, amino acid ethylester, thymine, ATP, RNA as well as DNA remained quite indifferent to 4-nitroquinoline N-oxide, under the same condition in which SH-group easily reacts with this compound. However, it is not possible to conclude that 4-nitroquinoline N-oxide does not react with important cell components other than SH-group.

What reactions actually take place *in the cell* must be elucidated by further experiments.

Summary

The substitution reaction between the nitro-group of various quinoline derivatives with SH-group was investigated under physiological conditions *in vitro*, and a definite relation was shown to exist between the carcinogenic action and reactivity of the nitro-group of these nitroquinoline N-oxides.

It was also shown that no reaction took place between 4-nitroquinoline N-oxide and amino acids, nucleotides as well as nucleic acid under the same experimental conditions, under which the nitro-group of this quinoline N-oxide so readily reacted with SH-group.

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要　　旨

4-Nitroquinoline N-oxide 誘導体の癌原性とそのニトロ基の SH 化合物に対する反応性との相関関係

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癌原性および非癌原性の7種の Nitroquinoline N-oxide (N-oxide を欠くもの一種，と Pyridine 誘導体一種を含む) についてそのニトロ基の SH 化合物に対する交換反応活性度を測定したところ癌原性発現と活性度との間に一定の関係のあることが見出された。

また同一の条件でそのニトロ基のアミノ酸，ヌクレオチド，及び核酸に対する反応性を観察したが，反応は陰性であった。

(文部省科学研究費による)

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